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METABOLISM OF SUCROSE BY STREPTOCOCCUS SANGUIS 804 (NCTC 10904)
AND ITS RELEVANCE TO THE ORAL ENVIRONMENT

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The work described in this thesis was carried out in the Department of Dental Biochemistry since October 1974. None of this work has been submitted in any other thesis or for any other degree.

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Symbols and Abbreviations

CHO↓	Precipitable carbohydrate (polysaccharide)
ECF	Extracellular fluid (of dental plaque)
FT	Fructosyltransferase
Fru	Fructose
Gal	Galactose
Glc	Glucose
GT	Glucosyltransferase
HA	Hydroxylapatite
K_m^{app}	Apparent K_m

SUMMARY

The extracellular glucosyltransferases of Streptococcus sanguis polymerise the glucosyl moiety of sucrose to form high molecular weight complex glucans. The adhesive and agglutinative properties of these glucans are important in the formation of dental plaque and, hence, in cariogenesis.

The glucosyltransferases of S. sanguis 804 (NCTC) were extensively purified (182-fold) by hollow fibre ultrafiltration (Bio-Fiber 80) followed by ammonium sulphate precipitation (0-70% of saturation). The enzymes were further purified by hydroxylapatite chromatography and appeared by this technique to consist of at least three enzymes with differing specific activities. It is not known whether these enzymes are, in fact, composed of different polypeptides or are modified forms of one protein.

The activity of the glucosyltransferases can be measured as the rate of release of fructose from sucrose or as the rate of synthesis of ethanol-sodium acetate precipitable polysaccharide (glucan). Using the former method, K_m^{app} for sucrose of $(NH_4)_2SO_4$ purified glucosyltransferases was about 6 mmol/l, and using the latter method, K_m^{app} was about 20 mmol/l.

Glucosyltransferase activity (as rate of glucan synthesis) was stimulated 2 to 4-fold by low concentrations (0.125-0.50 μ mol/l) of T2000 Dextran (Pharmacia; mol.wt. 2×10^6). Glucan synthesis was inhibited slightly by nigerose and was inhibited strongly by metrizamide (85% inhibition at 170 μ mol/l metrizamide). The rate of release of fructose was not affected by either xylitol or hydrogen peroxide.

The rate of synthesis of precipitable glucan was strongly inhibited by high concentrations of substrate (sucrose); the rate of release of fructose was relatively unaffected. The proposed mechanism for this effect is that sucrose acts as an alternative glucosyl acceptor (as well as donor) and thus inhibits glucosyltransfers to growing glucan chains.

The oral concentrations of sucrose during and after consumptions of various sweet foods and beverages were studied and were often sufficient to inhibit glucan synthesis. In such cases, the sucrose concentrations for maximum rate of glucan synthesis only occurred as sucrose was cleared from the mouth, after the food or drink was finished. Glucan synthesis by S. sanguis is important in plaque formation. Thus, these results provide an additional explanation for the clinical finding that the incidence of caries is related to the frequency of dietary intake of sucrose and not merely the total amount of sucrose consumed.

TWO ASPECTS OF DENTAL CARIES

INCIDENCE

'Caries is a scourge of civilisation; not until the progress of civilisation had brought about dietary refinements did this disease occur in human history in such terrifying proportions.'

Prof. J.-G. Helmcke (1971)

EFFECTS

'My curse upon your venom'd stang,
That shoots my tortur'd gooms alang
An' thro' my lug gies monie a twang
Wi' gnawing vengeance;
Tearing my nerves wi' bitter pang
Like racking engines.'

Robert Burns (1795/1796),

in 'Address to the Toothache.'

I : INTRODUCTION

1.1 DENTAL CARIES

1.1.1 Epidemiology of dental caries

Dental caries is the most prevalent human disease in Westernised countries. This may seem surprising but, certainly, few 'Westerners' completely escape this disease. It affects more than 99% of the population and may be considered epidemic. Keene et al. (1971) and Rovelstad (1967) found that, between 1960 and 1969, only 0.2% of 565,489 and 45,936 U.S. naval recruits, respectively, were completely caries-free. Similarly, from 1,719 Danish Army recruits, only 0.17% were caries-free (Antoft, 1974). Moreover, the caries-free subjects were not noticeably different in terms of oral hygiene, or in dietary intake of sucrose or other carbohydrates. Indeed their teeth accumulated 'normal' amounts of dental plaque, which is the aetiological factor in dental caries.

The higher caries rate in Western countries is almost certainly caused by extensive dietary intake of refined sucrose. In recent years, changes in sucrose consumption have been followed by corresponding changes in the incidence of caries (Grenby, 1971). When other sources of dietary energy are used, caries is rare. In Hunan province, in China, 75% of a sample of 14 to 27 year-old students were totally caries-free, and most of the remaining 25% had very little caries (Afonsky, 1951). Here, starch (as rice) was the main carbohydrate source and 'junk food' and 'snacks' were absent. The Eskimo's principal energy source used to be the fat (blubber) of marine mammals. Amongst these people, caries was almost unknown until the introduction of 'civilised'

diets, such as tinned foods, many of which contain sucrose as a sweetener or preservative (Helmcke, 1971).

Patients with hereditary fructose intolerance lack hepatic fructose-bisphosphate aldolase. They cannot metabolise fructose, and, if they ingest fructose or fructose-containing sugars, such as sucrose, they become severely ill. As a result, they avoid foods containing sucrose. Newbrun (1969) showed that individuals with hereditary fructose intolerance had very little dental caries; 45% were completely caries-free. This too suggests that there is something distinctive about the metabolism of sucrose by dental plaque bacteria which leads to caries.

However, even in populations with little or no caries, periodontal disease is common (Bibby, 1970; Lunt, 1974) and is the major cause of tooth loss, especially in middle-aged and elderly people. Like caries, periodontal disease is also caused by microbial plaque, but, unlike caries, it may be produced by the plaque formed on a sucrose-free diet. The first stage of periodontal disease is marginal gingivitis in which the gum margins become inflamed and hypertrophied, and are prone to bleeding (Scopp, 1970). The inflammation spreads through surrounding bone and blood and lymph vessels. The collagen fibres of the periodontal membrane can then be attacked by invading bacteria. The gum margins recede and 'pockets' form, leading to 'mobility' (looseness) of the teeth. In extreme stages, the tissues suppurate and are exfoliated; this is the condition commonly termed 'pyorrhoea (alveolaris)' (Cohen, 1976). This destruction and necrosis of the supporting tissues results, eventually, in loss of the teeth.

1.1.2 Nature of dental caries

'Caries' is a latin word meaning 'decay' or 'rottenness'. In the clinical sense, it may refer to diseases of teeth or (formerly) of bone. In dental caries, the bacteria of dental plaque, a soft microbial integument on the tooth (see Section 1.2), destroy the dental enamel and then infect the underlying dental tissues, dentine and pulp, and the surrounding soft tissues of the periodontium (see Section 1.1.3).

The microbial aetiology of dental caries was first suggested over one hundred years ago (Erdl, 1843; Ficinus, 1847), although the actual mechanisms of microbial attack of teeth were not studied experimentally until about twenty years later (Magitot, 1867; Leber & Rottenstein, 1867; Underwood & Milles, 1881; Miller, 1890). Miller is often considered the father of modern cariology (study of dental caries). He showed that the micro-organisms which he found on the teeth fermented dietary carbohydrates, producing acids capable of dissolving enamel; amongst these, he specifically detected lactic acid. His 'chemico-parasitic theory' is the basis of modern theories of cariogenesis.

In spite of the amount and surprising sophistication of early work, the detailed mechanisms of cariogenesis are controversial and much of the biochemistry is still very ill-defined.

1.1.3 Theories on the formation of carious lesions

In the past, many theories have been put forward to explain the destruction of tooth structure, the disease now known as 'dental caries'. The Greek physician, Hippocrates (456 B.C.) proposed that tooth decay

was due partly to phlegms and partly to substances in foods. This theory was believed for a long time; Bourdet (1757), for example, believed that juices (both from food and body humours) were retained in the teeth, where they stagnated and caused decay. Galen (131 A.D.), physician to the Roman Emperor, Marcus Aurelius, suggested that deficiencies in the diet made teeth 'weak, thin and brittle'. This is known to be true, to some extent, if the diet is deficient in calcium or phosphorus, but his theory gave the impression that caries arose from within the tooth. This misconception also persisted for some time (Hunter, 1778). Galen and Hunter also suggested that excessive diets (i.e. over-eating) might cause 'inflammation' of the teeth.

One picturesque suggestion from the Middle Ages was the 'Worm Theory' of caries, whereby 'tooth-worms' burrowed into the teeth. Methods of treatment were devised and later workers claimed to have seen these worms (Pfaff, 1756)!

The first to describe the colonisation of teeth by micro-organisms was the Dutch pioneer of microscopy, Anthony van Leeuwenhoek. He showed that when teeth were scrupulously cleaned, 'puri et candidi' (clean and white), a gummy white matter ('materia alba') formed on them. He wrote:

'.... vidi dictae illi materiae inesse multa admodum
exigua animalcula jucundissimo modo se moventia'

('....I saw that there were, in the aforementioned (white) matter, many tiny living animalcules moving in a most sprightly manner') (Leeuwenhoek, 1683). He also gave an idea of the large numbers of these animalcules present on the teeth;

'.... a good thousand of 'em in a quantity of this material
that was no bigger than a hundredth part of a sand-grain.'

(Leeuwenhoek, 1683) but failed to recognise that they might be involved in caries.

Pfaff (1756) was the first to suggest that tooth decay was due to 'remains of food which undergo putrefaction between the teeth'. The idea developed that acids, formed from breakdown of food, attacked the teeth. However, fermentation and putrefaction were then believed to be chemical processes and the microbial aetiology of caries was not proposed until much later (Erdl, 1843; Ficinus, 1847). Miller (1890) has reviewed these and other theories of cariogenesis popular in the nineteenth century and earlier.

The following are some more recent theories, also disfavoured.

(1) Proteolytic theory

Gottlieb (1947) suggested that proteases attacked and weakened the enamel matrix. Although bacterial collagenases may attack dentine, this is probably not crucial in destruction of enamel.

(2) Proteolysis-chelation theory

Schatz and Martin (1962) suggested that enamel minerals (mainly calcium and phosphate) are removed by chelation with the products of bacterial metabolism of the organic matrix of the tooth. These products would include those formed by proteolysis (i.e. amino acids), as suggested by Gottlieb (1947).

(3) Phosphatase theory (Phosphoprotein theory)

This theory (Kreitzman et al., 1969; Kreitzman, 1974) suggests that the phosphoric acid produced by the action of bacterial phosphatases, attacks the hydroxylapatite of enamel. Such enzymes are certainly present in plaque, but there is no evidence to suggest that they are involved in dissolution of hydroxylapatite.

There is little evidence to support any of these theories and other theories are even less well founded. [These have been reviewed by Dreizen (1976)]. The evidence for and against the proteolysis and proteolysis-chelation theories has been discussed by Jenkins (1971; 1978, pp. 427-429).

The currently accepted theory of cariogenesis is based on W.D. Miller's (1890) original chemico-parasitic theory of acid destruction of enamel. Indeed, it has been rightly said that '.... his concepts have never been superceded, only amplified' (Burnett & Scherp, 1968, p.v). (Acid production by plaque bacteria and its effects on dental enamel are discussed, in detail, in Section 1.3.1).

When plaque is sufficiently thick, bacterial metabolism, in the depths of plaque, is anaerobic and large amounts of acid are produced from dietary carbohydrates (see 1.3 and 1.3.1). This produces deep lesions of the enamel in which the organic matrix of enamel is exposed. The organic material is affected only well after the onset of demineralisation and bacteria invade the lesion about the same time as changes are observed in this organic material (Darling, 1956a & b, 1959 and 1970). Within carious lesions, the pH is lower than on the tooth surface, and the pH decreases with increasing depth (Dirksen et al., 1962 and 1963). This is probably because the depths of these lesions are not accessible to rinsing or buffering by saliva; the pH remains low long after carbohydrate has been cleared from the mouth (Dirksen et al., 1962). At the bottom of the deeper lesions, the pH is often below 4.0 (Dirksen et al., 1963). At this pH, streptococci are no longer metabolically active but many lactobacilli, such as L. acidophilus, can still produce acid. Lactobacilli predominate at the 'advancing

front' of a carious lesion (McKay, 1976; Shovlin & Gillis, 1969) and will cause further demineralisation of the enamel within the lesion, subject to availability of fermentable carbohydrate. The invading bacteria enter the dentinal tubules and the collagenous matrix of the dentine may then be attacked by collagenase-like enzymes (and possibly other proteases) produced by the plaque micro-organisms (Makinen, 1970; Larmas, 1972). Once bacteria have broken down the enamel and dentine, they may attack the roots of the teeth and the periodontium. These tissues become inflamed and ultimately necrotic.

An 'abcess' is the inflamed pocket of pus which then accumulates at the root apex following bacterial infection. By this stage, the tooth may be non-vital or may even have been lost altogether. The periodontal tissues are inflamed and necrotic (see 1.1.1). Thus, the consequences of tooth decay are more than just 'toothache' and there can be a serious risk of bacterial infection of various other tissues if active caries is allowed to proceed unchecked.

1.2 Dental Plaque

'.... the disintegration begins on the outer surface of the enamel, and the one thing necessary to the beginning of caries is the formation of a gelatinous microbial plaque in a secluded position where its acids may act without too frequent disturbances, as in pits, fissures, approximal surfaces, about the gum margins, etc., and there give rise to caries'.

Black (1898)

1.2.1 Nature of plaque

The term 'plaque', to describe the acquired layer of bacteria and their surrounding matrix, on the tooth surface, was first used by Black, in 1898. Gibbons and Van Houte (1973) defined dental plaque as 'dense, non-calcified bacterial masses so firmly adherent to the tooth surface that they resist wash off by salivary flow.' To the naked eye, dental plaque is a thick, adhesive, cream-coloured film on the enamel surface. It has a gelatinous appearance, especially in subjects on a sucrose-rich diet (Carlsson & Egelberg, 1965). It accumulates mainly in the pits, fissures and around the gingival margin of the tooth.

The gross appearance of plaque can vary subtly from subject to subject. Microscopically, however, plaque is extremely heterogeneous. Even within one mouth, different sites may vary in both microbial composition (Ikeda & Sandham, 1971; Bibby, 1938; Critchley, 1969) and metabolism (Kleinberg & Jenkins, 1964).

In spite of this diversity, plaque has many regular features. For example, the predominant bacteria in human plaque, especially early

plaque, are certain non-haemolytic streptococci, mainly Streptococcus mutans, S. sanguis and S. miteor (from various authors, compiled in Gibbons & Van Houte, 1973; also Ritz, 1967). Other bacteria, such as S. salivarius, Actinomyces, Lactobacillus and Veillonella, are commonly found. Spirochaetes, Clostridium, Nocardia and yeasts (Candida spp.) may be present in small amounts (Socransky, 1970; Loesche et al., 1972; Nolte, 1973). (The microbial ecology of plaque and other oral structures and fluids has been extensively reviewed by Nolte, 1973.)

All dental plaques, regardless of composition, can produce acids by fermenting dietary carbohydrates. This was first shown by Miller (1890) and later by others (e.g. Muntz, 1943; Jenkins & Kleinberg, 1964; Gilmour & Poole, 1967). All plaques have the ability to produce extracellular polysaccharides (glucans and fructans) from dietary sucrose (Carlsson, 1965). Both of these properties are believed to be essential for cariogenesis by plaque bacteria.

It is now clear that the concentrations of acids, produced by fermentation in plaque, are sufficient to dissolve the hydroxylapatite of dental enamel under physiological conditions (Jenkins, 1966; see also Section 1.3.1).

Gibbons et al. (1966) showed that cariogenic strains of oral streptococci produced large amounts of extracellular polysaccharide from sucrose, while strains producing less polysaccharide were non-cariogenic. Mutant strains of S. mutans, defective in extracellular glucan synthesis, are (with respect to parent strains) less able to form plaque (Sharma et al., 1975; Bulkacz & Hill, 1977) and less cariogenic (Tanzer et al., 1974). Greer et al. (1971) found that cariogenic strains of S. mutans and S. salivarius were lysogenic, but that non-cariogenic strains were not.

Klein et al. (1975) reported that, when lysogenic strains of S. sanguis and S. mutans are 'cured', synthesis of extracellular polysaccharides (water soluble and insoluble) and plaque-forming ability both decrease. The role of the prophage in maintaining these two activities is not yet known but this does suggest that such polysaccharide synthesis is important in plaque formation. One apparently anomalous finding from this report was that, although the 'cured' mutants of S. sanguis produced less plaque and polysaccharide than parental strains, they caused more carious lesions in gnotobiotic rats. Klein et al. (1975) suggested from this that extracellular polysaccharide synthesis is not essential for cariogenesis. However, in gnotobiotic rats, mono-infected with S. sanguis, extracellular polysaccharide formation is probably not important for colonisation of the teeth, since S. sanguis can bind to pellicle and enamel (see Section 1.2.4). (With the mutants, less polysaccharide is synthesised, so there may be more sucrose 'available' for acid production.) Under 'normal' conditions other micro-organisms (e.g. S. mutans) will be present and extracellular polysaccharide formation will facilitate colonisation of plaque and cariogenesis, by these other species (see 1.2.4 and 1.2.5).

1.2.2 Formation of pellicle

When dental enamel is thoroughly cleaned of all extrinsic matter (e.g. by pumice abrasion), in situ, it is rapidly coated with a thin acellular film of organic material, 1-10 μm thick. This was termed 'acquired pellicle' by Dawes et al. (1963). Various workers showed that this pellicle is largely composed of salivary proteins (Turner, 1958a & 1958b; Meckel, 1965).

The mechanism of pellicle formation is unclear. Ericson (1967) found that certain salivary glycoproteins, especially those rich in sialic acid residues, were strongly and specifically bound to hydroxylapatite, in vitro. Most of the salivary proteins which were absorbed to hydroxylapatite and powdered enamel were rich in proline, glycine and dicarboxylic amino acids (Armstrong, 1971; Hay, 1973). The importance of the proline content is not clear. Sönju and Rølla (1972a & b, 1973) showed that the protein of 2 hour-old pellicle was low in sulphur-containing and basic amino acids (including proline) but was rich in acidic amino acids. The amino acid composition of the pellicle on different tooth surfaces varied little (Sönju & Rølla, 1973). From these findings it has been proposed that pellicle is formed mainly by selective absorption of acidic salivary proteins to enamel, possibly assisted by Ca^{2+} ion bridging (Cole & Eastoe, 1977, p.376; Jenkins, 1978, p. 368). The absorption appears to be selective, because while blood group substances and inhibitors of viral haemagglutinins are adsorbed from saliva, α -amylases, the major proteins in saliva, are not (Sönju et al., 1974).

Neuraminidases from plaque and salivary bacteria can precipitate salivary protein by removing sialic acid residues and this may be involved in formation of the extracellular matrix of plaque (Leach, 1963; Fukui et al., 1971). White (1976) has proposed that this is also involved in pellicle formation. This remains to be proved, but it seems unlikely since the sialic acid would be more likely to aid adsorption of the proteins to the enamel.

There have been few attempts to identify the protein components of pellicle. Ørstavik and Kraus (1973), using immunofluorescence, found that IgA and lysozyme were present in most pellicles; IgG and α -amylase

occurred occasionally and fibrinogen and albumin were rare. However, as mentioned above, Sonju et al. (1974), using biochemical and immunological assays, found that blood group substances and haemagglutination inhibitors were present but that lysozyme and α -amylase were not.

Whatever the detailed mechanisms of formation, pellicle does accumulate uniformly on tooth surfaces and is important in the formation of dental plaque (see 1.2.4).

It has been suggested that the function of pellicle is to protect the enamel. In support of this, pellicle does appear to slow down solubilisation of enamel by acid (Darling, 1943; Meckel, 1965). However, this question is still unresolved, and the harmful effects of pellicle, in facilitating binding of bacteria to enamel (see 1.2.4), probably outweigh any such benefits.

1.2.3 Colonisation of teeth by bacteria

On a clean or pellicle-coated tooth surface, clumps of bacteria appear within a few hours, either by growth from the pre-existing microflora or by colonisation from oral fluids (Saxton, 1973; Björn & Carlsson, 1964). These clumps eventually grow, coalesce and build up a layer of bacteria, polysaccharide and other matter, up to 300 μ m thick. This is dental plaque.

The mechanisms of plaque formation are not fully understood. Various factors have been implicated in colonisation of teeth by bacteria. Low pH and agglutination by ion bridging or by proteins from saliva and preformed plaque ~~have been suggested~~ (Silverman & Kleinberg, 1967; Gibbons & Spinell, 1970; Jenkins, 1978, p.378). All of these probably contribute to plaque formation but the importance of extracellular glucans in

bacterial adhesion is rapidly becoming apparent (see 1.2.4).

S. mutans is not abundant in saliva and probably colonises tooth surfaces by migration and growth from small plaque deposits remaining in tooth pits and fissures and on the gingivae; S. sanguis and S. miteor, on the other hand, are more abundant in saliva than S. mutans is. They probably colonise the teeth by absorption from saliva (Gibbons & Van Houte, 1973). Such differences partly explain why thorough removal of bacteria from the teeth and gingivae considerably (but only temporarily) alters the microbial population of dental plaque.

1.2.4 Adhesion of micro-organisms to the tooth surface

In dental plaque, the predominant bacterial species are streptococci, mainly S. mutans, S. sanguis and S. miteor, with traces of S. salivarius and S. milleri (see 1.2.1). The aggregation and adhesion properties of these species differ.

S. sanguis and S. salivarius are aggregated by saliva (Kashket & Donaldson, 1972). S. mutans is not, but is specifically agglutinated by various glucans, including dextran[‡] (Gibbons & Fitzgerald, 1969; Guggenheim & Schroeder, 1967) and its own 'mutan'[‡] (Veld & de Stoppelaar, 1975). In vitro, all these species bind poorly to uncoated enamel, but S. sanguis binds strongly to pellicle-coated enamel (Ørstavik et al., 1974). Also in vitro, S. sanguis and S. miteor bind to saliva-coated (i.e. quasi-pellicle-coated) hydroxylapatite (HA), but not to dextran coated HA; S. mutans binds only to dextran-coated HA (Liljemark & Schauer, 1975). In the same study, the binding abilities of S. sanguis and S. mutans were destroyed by pre-treatment with proteases, suggesting that both types of binding site are, or contain, protein. The binding of S. mutans was also sensitive to pre-treatment with dextranase.

[‡]See Appendix

Ørstavik (1978) has recently attempted to identify the pellicle components to which S. sanguis binds; some of the components were isolated and shown to be proteins. Of these, only lysozyme was clearly identified. Lysozyme alone could not promote adhesion so S. sanguis may have to bind simultaneously to several receptors (or proteins?).

Mukasa and Slade (1973, 1974a & 1974b) showed that adhesion of S. mutans to glass required the activity of glucosyltransferases adsorbed to the cell surface. This was confirmed by the finding that this sucrose-dependent adhesion was inhibited by dextranase, which prevented production of cell-surface glucans (Schachtele et al., 1975). However, this property is probably independent of the glucan-induced agglutination of S. mutans, since soluble dextrans inhibit both the enzyme activity (Newbrun et al., 1977) and adsorption to the cell surface (Mukasa and Slade, 1974a) of these glucosyltransferases. Cell-bound glucosyltransferase activity and dextran-induced agglutination can each be abolished without affecting the other (McCabe & Smith, 1975). McCabe et al. (1977) isolated, from a cariogenic strain of S. mutans, an extracellular dextran-binding protein which had no glucosyltransferase or dextranase activity; the evidence is, admittedly, circumstantial, but this protein may be a receptor involved in dextran-induced agglutination and adhesion to the tooth surface. Finally, Kuramitsu (1973) showed that both live and heat-killed S. mutans cells bound to dextran-coated glass; binding was independent of any glucosyltransferase or other heat-labile enzyme activity.

The importance of S. mutans cell-bound glucosyltransferases in plaque formation is not clear. Although glucan-induced agglutination is important, there are certainly other factors involved. Non-plaque-

forming mutants of S. mutans which can still be agglutinated by dextran, have been isolated (Freedman & Tanzer, 1974). Freedman and Tanzer argue from these findings that agglutination is not involved in plaque formation at all. However, they used a model system, in vitro, with 'plaque' formation on metal wires, in which even wild-type S. mutans probably binds by other mechanisms anyway. In vivo, tooth colonisation by S. mutans is probably facilitated by the glucans of other bacteria such as S. sanguis; moreover, glucan-induced agglutination will, in vivo, promote 'clump' formation, which will also accelerate plaque formation by S. mutans.

The colonisation of teeth by S. mutans is very dependent on dietary sucrose levels, while colonisation by S. sanguis is not (de Stoppelaar et al., 1970). This suggests that adhesion of S. mutans is facilitated by an enzyme (or enzymes) using sucrose as substrate and is not merely due to passive agglutination.

Less is known about the adhesion, in plaque, of other bacterial species. Actinomyces viscosus is aggregated by Leuconostoc mesenteroides dextran and the extracellular glucans of S. sanguis and S. mutans, and binds strongly to Sephadex (cross-linked L. mesenteroides dextran gel) (McBride, 1975). Many Actinomyces species synthesise adhesive dextran-like glucans from sucrose, but can also form plaque in the absence of sucrose (Jordan et al., 1969). These extracellular glucans may facilitate adhesion of Actinomyces to other species of bacteria in plaque. It has been shown, with the scanning electron microscope, that, in plaque, cocci co-aggregate with filamentous (Actinomyces-like?) bacteria giving a 'corn-on-the-cob' appearance (Gibbons & Van Houte, 1973). Veillonella, a fairly common plaque bacterium, co-aggregates with

A. viscosus, in vitro (Gibbons & Nygaard, 1970). Also, Veillonella alone cannot form 'plaque' on metal wires, but can bind to pre-formed 'plaques' of A. viscosus (Bladen et al., 1970).

Wittenberger et al. (1977) showed that the glucosyltransferases of S. salivarius bind very strongly (i.e. not removable by 1.0 mol/l NaCl or LiCl) to the surface of V. parvula cells. The effect of this (if any), in vivo, is not yet known, but it may enhance adhesion of V. parvula to smooth surfaces, and interspecies aggregation.

Candida spp. (e.g. C. albicans) are occasionally present in plaque. They may infect the soft tissues (giving candidiasis) but it is not known if they are involved in cariogenesis. Adhesion of Candida to dental prostheses ('false teeth') is sucrose-dependent (L.P. Samaranayake, personal communication) but the mechanism of this is not yet known.

In conclusion, the binding of bacteria to enamel and pellicle is important in plaque formation. The ability of different species to bind to each other is certainly just as important.

1.2.5 Current model for dental plaque formation

Using the information given above (Section 1.2.4), one can construct a model to describe the formation of dental plaque. The salient points are the following.

- (1) Plaque streptococci and other bacteria produce adhesive extracellular glucans from dietary sucrose.
- (2) S. mutans cells are agglutinated by extrinsic dextrans or by glucans produced by their own cell surface enzymes.
- (3) S. sanguis and S. miteor can bind to enamel or, better, to pellicle-coated enamel.

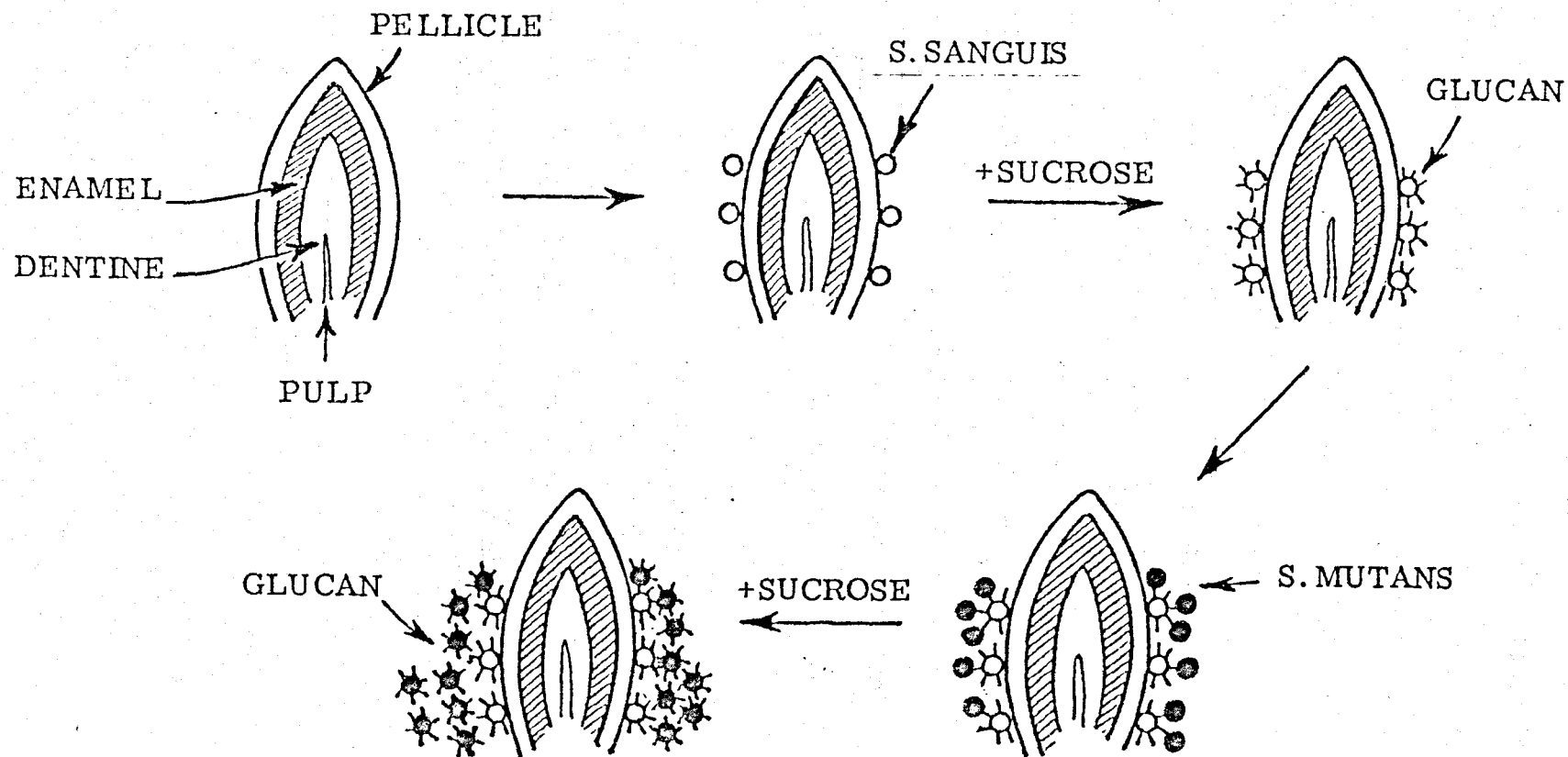
- (4) The colonisation of teeth or plaque by S. mutans requires the presence of sucrose. Colonisation by other species, such as S. sanguis and A. viscosus, does not.
- (5) Many plaque bacteria are aggregated by glucans or by inter-species adhesion.

Therefore, when the surface of enamel is cleaned, pellicle rapidly forms and S. sanguis, S. miteor, and possibly other species (e.g. Actinomyces spp.), bind to it. In the presence of sucrose, these bacteria synthesise extracellular glucans which facilitate attachment of S. mutans and other bacteria. Glucan synthesis continues and more bacteria bind to the surface of the growing plaque. (The essentials of this scheme are summarised in Fig. 1.1.)

Some workers, however, doubt the importance of plaque glucans as agglutinating, adhesive factors in the formation of dental plaque. Jenkins (1978; pp.386-387) points out that, although 'dextrans' bind to hydroxylapatite, the binding may be inhibited by pellicle formation and is certainly inhibited by physiological (i.e. plaque and salivary) concentrations of inorganic phosphate (Rölla & Mathiesen, 1970). However, this does not take into account the roles of S. sanguis, S. miteor and other glucan-producing species. With the scheme described above, in the presence of these species, it is not necessary for S. mutans to bind directly to the tooth surface, whether glucan-coated or not.

Glucan-induced agglutination and glucosyltransferase-mediated adhesion of S. mutans are independent activities (see 1.2.4) and this has led to the suggestion that agglutination is not involved in adhesion (or, hence, in plaque formation) by S. mutans. Preliminary work by

FIG. 1.1 FORMATION OF DENTAL PLAQUE



McCabe and co-workers (cited by McCabe, 1976) suggests that the two may be distinct properties mediated by a single receptor, possibly the 'dextran-binding protein' isolated by McCabe et al. (1977). They propose that agglutination is due to binding of soluble glucan to this receptor while adhesion is due to syntheses of complex, insoluble glucans bound to the receptor and to other surfaces. These complex glucans might be produced by S. mutans cell-bound enzymes or by pellicle-bound bacteria of early plaque (e.g. S. sanguis).

Freedman and Tanzer (1974) isolated mutants of S. mutans, which were unable to form 'plaque' in vitro. They concluded that glucan-induced agglutination was not involved in plaque formation by S. mutans, in vitro. However, as pointed out in Section 1.2.4, this probably is not the case in vivo. Certainly in vitro and in germ-free animals, S. mutans (wild-type) probably binds to teeth by other mechanisms, such as by synthesis of its own cell-surface glucans, as suggested by Mukasa and Slade (1973).

Finally, in further support of the hypothesis that S. sanguis and S. miteor bind to pellicle in formation of early plaque, it has been shown that S. sanguis and S. miteor are recovered in higher proportions from very early plaque than are other species such as S. mutans (Carlsson, 1965).

1.3 Metabolism of Sucrose by Dental Plaque

'... Sweet, sweet, sweet poyson of the age's tooth.'

William Shakespeare (1596/1597) in 'King John' I, i, 1.213

Because dental plaque is so heterogeneous, it contains a wide range of metabolic systems. Our main concern is the metabolism of sucrose, which is the single most cariogenic component of the diet.

Sucrose can be metabolised by plaque bacteria in the following ways:

- (i) hydrolysis by invertase to glucose and fructose followed by
 - (a) catabolism to CO_2 and/or organic acid,
 - (b) anabolism to glycogen-like intracellular polysaccharide
- (ii) synthesis of extracellular fructans (with release of glucose),
- (iii) synthesis of extracellular glucans (with release of fructose).

It has long been known that plaque bacteria can anaerobically produce acids which might attack dental enamel (Miller, 1890; Muntz, 1943). Moreover, many of the bacteria isolated from plaque are facultatively or obligately anaerobic (Loesche et al., 1972; Ritz, 1967; Socransky, 1970). It used to be thought, and is still widely believed (Kleinberg, 1970; Mandel, 1974; Saxton, 1975; Tatevossian and Gould, 1976), that dental plaque was a barrier to diffusion of various ions and molecules (including oxygen). However, the work of McNee et al. (1979) has shown that oxygen and other small molecules can diffuse freely through plaque at about half the rate found in water. If oxygen diffuses into 100 μm plaque, the time required to reach half the equilibrium concentration ($T_{\frac{1}{2}}$) is only about 1.9s (McNee et al., (1979). Through 100 μm of water, $T_{\frac{1}{2}}$ is about 0.9s (calculated from

data in Reid and Sherwood, 1958, p.554 and Daniels and Alberty, 1966, p.384). This makes the presence of anaerobic bacteria, in plaque, superficially surprising. However, the most likely explanation for the occurrence of anaerobic conditions in plaque is that oxygen is metabolised very rapidly by the surface micro-organisms of the plaque. Thus, the plaque must be fairly thick and the metabolic rate must be high enough to prevent more than superficial penetration of the plaque by oxygen.

(Acid production by plaque is discussed further in Section 1.3.1).

Sucrose and other soluble di- and mono-saccharides are rapidly cleared from the mouth. They can be stored by plaque bacteria as extracellular glucan or fructans (see 1.3.2 and 1.3.3) or as intracellular iodophilic polysaccharides. The latter are glycogen-like and are synthesised by most plaque bacteria (Gibbons & Socransky, 1962; Berman & Gibbons, 1966; van Houte & Jansen, 1968a and 1969). The bacteria synthesise this 'glycogen' during periods of high oral sugar concentration or even during 'resting' phases. In the absence of other nutrients, the 'glycogen' is broken down to form acids.

The glucans (other than glycogen) and fructans of plaque are synthesised, by extracellular or cell-bound enzymes, from sucrose. These are discussed in Sections 1.3.2 and 1.3.3.

1.3.1 Acid production by dental plaque

Miller (1890) was the first to show that micro-organisms in dental plaque could produce acids by fermenting dietary carbohydrates. This has since been verified by numerous workers (e.g. Stephan, 1938; Carlsson, 1965; Muhlemann and de Boever, 1970).

Using antimony electrodes, Stephan (1940) showed that the pH of undisturbed plaque decreased when the mouth was rinsed with glucose or sucrose. The pH dropped rapidly to a minimum (as low as 4.0) within 10 minutes and slowly returned to the 'resting' level by 30 to 60 minutes after rinsing. (The graph of plaque pH against time is now known as a 'Stephan curve'.) Initial (resting) pH values and pH minima were lower in caries-active subjects and only in these subjects did pH drop below 5.0 (Stephan, 1943; Jenkins and Kleinberg, 1964).

The pH at which the hydroxylapatite of enamel dissolves (the so-called 'critical pH'), is lowered by increased calcium and inorganic phosphate concentrations in the surrounding media. Saliva contains about 1.5 mmol/l calcium and about 6 mmol/l inorganic phosphate (Gow, 1965; Tatevossian and Gould, 1976). In this environment, enamel is decalcified below pH 5.5 (Jenkins, 1978; p.299). The concentrations are somewhat higher in plaque extracellular fluid (ECF) [calcium, 6.5 mmol/l, and inorganic phosphate, 14.2 mmol/l; Tatevossian and Gould (1976)] but hydroxylapatite is still solubilised below pH 5.5, even at these concentrations.

More convincing, perhaps, than sugar rinses, are Stephan curves and lactic acid production, in plaque, after ingestion of carbohydrate-containing foods and drinks (Ludwig and Bibby, 1957). The Stephan curves resemble those from glucose rinses, although the time-courses vary between foods. This technique has been used to assess the relative 'acidogenicity' of various snack foods (Edgar et al., 1975; Rugg-Gunn et al., 1977). This is important because the snack habit probably greatly increases the incidence of dental caries.

However, the pH of plaque depends on more than just the fermentability of dietary carbohydrates. Old or mature plaques give

lower pH minima following carbohydrate rinses, partly because of the greater amount of anaerobic bacteria in deep plaque (de Boever and Muhlemann, 1970). The amount of acid produced also depends on the availability of fermentable carbohydrate. For example, dissolved carbohydrates will be more rapidly metabolised than insoluble ones.

Stephan (1940) showed the drop in pH in plaque was, within limits, proportional to the concentration of glucose in the rinses. However, 'salivary sediment' produced slightly less acid with glucose above 100 g/l (0.555 mmol/l) (Sandham & Kleinberg, 1969). Similarly, at high concentrations of sucrose (above 0.1 mmol/l) plaque produced less acid and shallower Stephan curves were obtained (Geddes, 1974 and 1975; Birkhed & Frostell, 1978). This may be a bacteriostatic physical effect (e.g. osmotic) or may be due to excess substrate inhibition of bacterial enzymes. Invertase and dextransucrase of other micro-organisms are known to be inhibited by high sucrose concentrations, although probably for different reasons with each enzyme (Nelson & Schubert, 1928; Hehre, 1946). This may be a common feature of sucrose-metabolising enzymes.

The lowering of pH in plaque is affected, not only by the carbohydrate content of foods, but also by their physical nature and texture. Some foods are cleared from the oral cavity more rapidly than others because of differences in solubility or adhesiveness. Moreover, most foods will contain various buffers which will help minimise pH changes.

The magnitude of the drop in pH due to acid production is also affected by the chemical nature of the acids (i.e. their pK's), by

dilution of acids by salivary flow and by buffers in saliva, in plaque extracellular fluid (ECF) and in food. The pH drop produced in human plaque by sweet foods can be 'buffered' by eating non-acidogenic savoury foods such as cheese or peanuts immediately afterwards (Rugg-Gunn et al., 1975, 1978; Geddes et al., 1977). Such foods stimulate salivary flow, which helps dilute the acids produced by plaque. They also contain large amounts of protein which may be metabolised by plaque bacteria to produce ammonia. Many cheeses (e.g. blue cheese) contain ammonia as a product of fungal metabolism. This ammonia raises the pH of the plaque. Thus, the Western custom of eating sweet foods at the end of meals is the worst possible one insofar as dental health is concerned. The Chinese eat sweet foods, ad libitum, throughout the course of a meal. This seems more desirable.

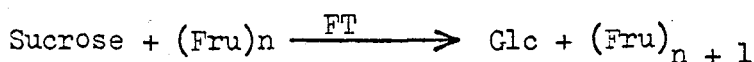
As mentioned previously, plaque bacteria can ferment carbohydrates to acids, in vitro (Miller, 1890; Muntz, 1943). Others have since demonstrated this in vivo. Moore et al. (1956) studied the pH and lactate concentrations of human plaque, in situ. The drop in pH following sucrose rinses was paralleled by increases in concentration of lactate. Moore and co-workers claimed that the drop in pH was fully accounted for by the lactate produced and that lactate was the only acid produced. However, Strålfors and Eriksson (1959) have shown that other acids in plaque could interfere with the method used for assaying total lactate and so lead to an over-estimate of lactate concentration. Using the more sensitive technique of gas-liquid chromatography, Geddes (1975) showed that plaque produced L(+)-lactate, D(-)-lactate and the volatile acids, acetate, propanoate and butanoate. The volatile acids predominated in 'resting' plaque, but, after ingestion of sucrose, the concentrations of L(+) and D(-) lactate increased rapidly.

These two are the major acid species at, and following the pH minima of Stephan curves. The pK of lactate (3.86 at 25°C) is much lower than those of acetate (4.76), propanoate (4.87) and butanoate (4.82). With Geddes' findings, this suggests that lowering of pH in plaque (and, hence, initiation of carious lesions) is mainly due to production of lactate.

1.3.2 Synthesis of plaque fructans

Fructan synthesis in dental plaque is less well characterised than glucan synthesis. The fructans in plaque are often loosely termed 'levans' (β -2,6-linked fructan with β -2,1-linked branches; see fig. 1.2) and the enzymes which synthesise them have been called 'levansucrases' (EC. 2.4.1.10; sucrose: 2,6- β -D-fructan 6-fructosyltransferase). This is mainly because many bacteria, such as Aerobacter levanicum and Bacillus subtilis synthesise levans (Feingold & Gehatia, 1957; Rapoport & Dedonder, 1966). Like the terms 'dextran' and 'dextransucrase', these may be misnomers when applied to plaque (see Appendix). Methylation analysis has shown that the fructans of S. mutans Ingbritt A are mainly β -2,1-linked (Baird et al., 1973) and thus are more like inulin (see fig. 1.3) than like levan. However, the infra-red spectra of Actinomyces fructans (Howell & Jordan, 1967) closely resemble that of levan, rather than inulin, as demonstrated by Barker and Stephens (1954).

Plaque fructans are synthesised by fructosyltransferases (FT) as follows:



The fructosyltransferases of S. mutans and A. viscosus can also use raffinose as fructosyl donor (Carlsson, 1970; Howell & Jordan, 1967).

The reaction is **exothermic** ($\Delta G^\circ = -8.4$ kJ/mole) but is less so

Fig. 1.2 ILEVAN

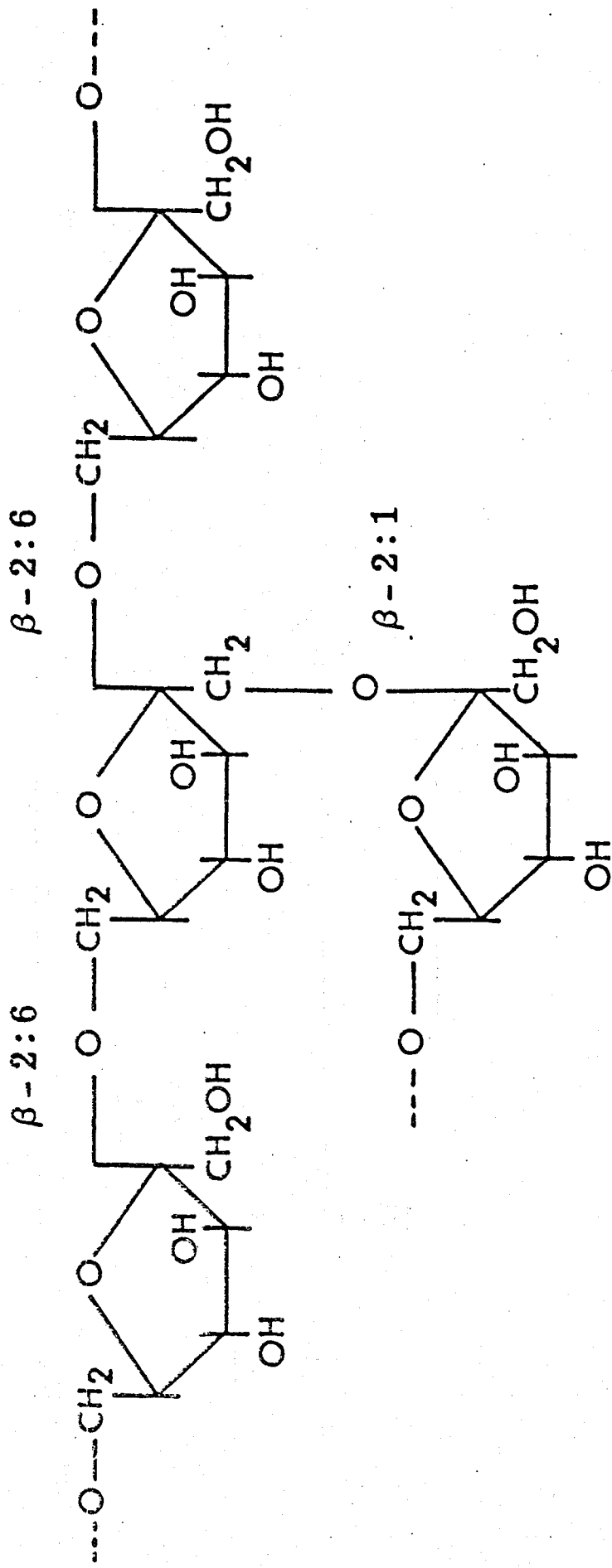
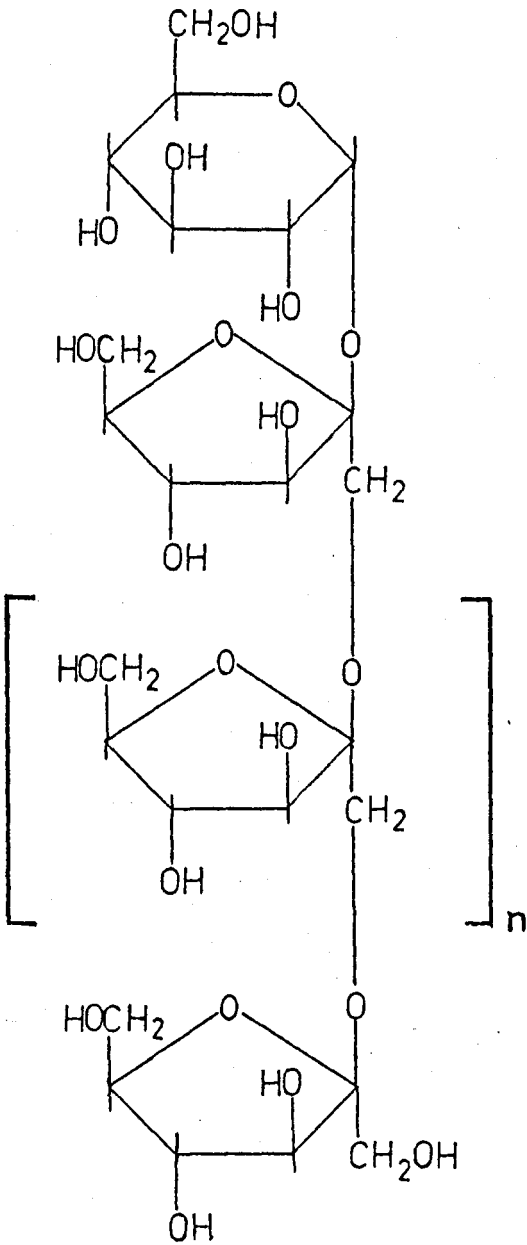


Fig. 1.3 INULIN



$[n \approx 29]$

than the reaction for glucan synthesis ($\Delta G^\circ = -116 \text{ kJ/mole}$, according to Cole & Eastoe, 1977, p.281).

Unlike plaque glucans, fructans are not 'sticky' and are readily water-soluble. Although fructans are synthesised more rapidly than glucans in plaque (Higuchi et al., 1970), their overall concentrations are lower because they are also rapidly degraded by plaque bacteria (McDougall, 1964; Wood, 1964 and 1967a; Wood & Critchley, 1967). Thus plaque fructans are probably carbohydrate 'stores' rather than adhesive matrix material, as is the case with glucan (McDougall, 1964; Da Costa & Gibbons, 1968; van Houte & Jansen, 1968a).

Fructans are synthesised by various plaque micro-organisms, such as A. viscosus (Howell & Jordan, 1967), S. mutans (Wood & Critchley, 1967) and S. salivarius (Garszczynski & Edwards, 1973) but not S. sanguis (Black, 1975).

Carlsson (1970) purified the fructosyltransferase activity of S. mutans and showed it to have a pH optimum of 6.0, pI of 4.2 and temperature optimum of about 40°C . Ca^{2+} appeared to increase enzyme activity (or at least stabilise the enzyme molecule). The fructosyltransferase of S. salivarius had a pH optimum of 5.6, a pI of 5.2, an apparent K_m for sucrose of 17 mmol/l , and molecular weight by gel filtration (on Bio-Gel P-60) of 34,500 (Garszczynski & Edwards, 1973; Whitaker & Edwards, 1976). The former claim that enzyme activity is inhibited by divalent cations, while the latter claim that it is stimulated and quote K_m for Mg^{2+} as $63 \text{ } \mu\text{mol/l}$. The reason for the difference in the findings is unclear. Scales et al. (1975) found that a 'glycosyltransferase complex' (containing glucosyltransferase and fructosyltransferase), from S. mutans FA1, was strongly inhibited by some divalent cations but was unaffected by Ca^{2+} or Mg^{2+} . The

fructosyltransferases of S. mutans and S. salivarius are constitutive (Carlsson, 1970; Garszczynski & Edwards, 1973). Those of A. viscosus are inducible by sucrose or raffinose (Howell & Jordan, 1967).

The function of plaque fructosyltransferases is to store carbohydrate. They are thus important in the production of acid after food has been cleared from the mouth. Given the low K_m of the enzyme from S. salivarius (17 mmol/l; see above), these enzymes are probably very active at normal dietary and oral concentrations of sucrose (see Results, section 3.7). The K_m is similar to values for the glucosyltransferases of S. sanguis and S. mutans (see 1.3.4). The K_m reported by Scales et al. (1975) is unusually high (55 mmol/l) but they too found that the values for fructosyltransferase and glucosyltransferase were identical. Aksnes (1977) found that the glucosyltransferase, fructosyltransferase and invertase of S. mutans co-focused as one symmetrical peak (at pH 4.6) in liquid column isoelectric focusing. He suggested that these different enzymes had a common apoenzyme. It is perhaps surprising that only one peak of enzyme activity was observed since S. mutans produces several glucosyltransferases (see 1.3.4) and its glucosyltransferases and invertase are distinct proteins (Fukui et al., 1974). The findings of Aksnes are not conclusive but, in view of this and the similarity of K_m 's, it is reasonable to suppose that the S. mutans enzymes at least possess some common sub-units, including, perhaps, the sucrose-binding site.

1.3.3 Structure of plaque glucans

Extracellular glucans are synthesised, from sucrose, by many plaque bacteria, such as Streptococcus spp. (Carlsson, 1965) and

Actinomyces spp. (Gibbons & van Houte, 1973). The molecular structures of the streptococcal glucans have been studied intensively by various methods. The results obtained are affected by the methods of analysis used, by the conditions and media for bacterial growth and by the properties of the glucans themselves. The structure of the glucans is also affected by the methods of production, whether synthesised by bacteria grown in the presence of sucrose or by purified or partially purified glucosyltransferases (Nisizawa et al., 1977; Ceska et al., 1972). These problems have been reviewed by Black (1975).

The extracellular glucans of dental plaque are often misleadingly referred to as 'dextran' (see Appendix). It is now clear that their structures are much more complex. Many different structures have been reported for the glucans of S. mutans and S. sanguis (Long, 1971; Long & Edwards, 1972; Baird et al., 1973; Sidebotham, 1974; Arnett & Mayer, 1975; Black, 1975; Krautner & Bramsted, 1975; Usui et al., 1975; Nisizawa et al., 1976; Beeley & Black, 1977; several other sources are described by Black, 1975, and Sidebotham, 1974). However, in spite of these variations in findings, it is clear that the streptococcal glucans contain predominantly α -1,6 and α -1,3 linkages, in both branches and chains, although the reported proportions of each linkage vary. The dextran of Leuconostoc mesenteroides contains only a small proportion of α -1,3 linkages, generally less than 10% of the total number of linkages (Sidebotham, 1974). However, the glucans of S. sanguis contain up to 48% α -1,3 linkages, and those of S. mutans, up to 84% (Guggenheim, 1970). The latter were shown to be α -1,3 linked glucans with α -1,6 branches. These 1,3- α -D-glucans are called 'mutan' and, unlike dextran, are insoluble in water.

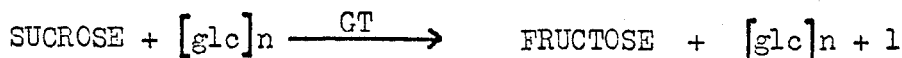
Stoudt and Hollstadt (1974) have reported an unusual plaque glucan. This was synthesised from sucrose, in the presence of dextranase,

by plaque formed in vitro, using S. mutans. The glucan was named 'cariogenan' and contained 75% α -1,3 linkages and 25% α -1,2 linkages in a linear (i.e. unbranched) molecule. An uncharacterised Bacillus glucanase hydrolysed cariogenan to glucose and nigerotriose [α -D-glc-1,3- α -D-glc-1,3- α -D-glc]. It may thus be an α -1,3 linked polymer of glucosyl- α -1,2-nigerotriose (see fig. 1.4). Cariogenan is very insoluble in water and so may be adhesive and important in plaque formation.

1.3.4 Synthesis of plaque glucans - glucosyltransferases

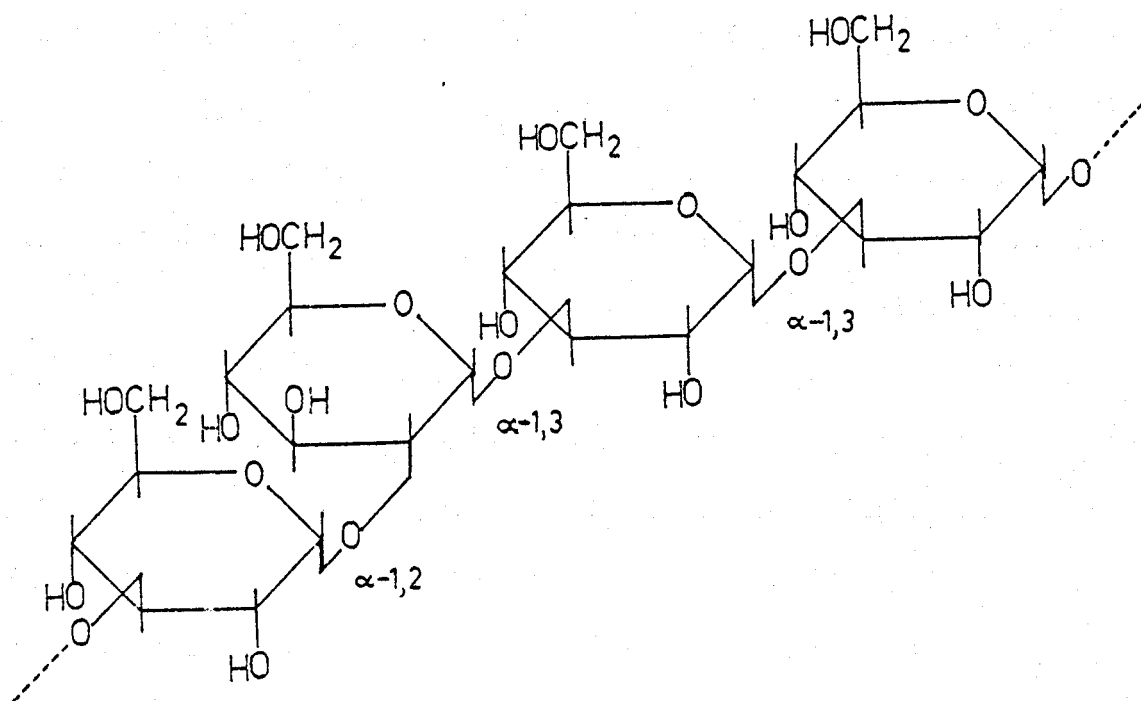
[This section should be read in conjunction with the Appendix at the end of the thesis.]

Extracellular glucans are synthesised from sucrose by glucosyltransferases (GT), as follows (Eisenberg & Hestrin, 1963):



The growing glucan chain acts as a glucosyl acceptor. Other compounds (see below) may also do so. The enzyme-catalysed reaction does not require synthesis of any high-energy precursors (such as UDP-glucose in glycogen synthesis) because of the high free energy of hydrolysis of sucrose [$\Delta G^{\circ'} = -29.3 \text{ kJ.mol}^{-1}$]. The free energy change is so large because the anomeric carbon atoms of the two hexose moieties (C-1 in glucose and C-2 in fructose) are both involved in the glycosidic link, making sucrose a diglycoside. The free energies of formation of the various glucans in plaque, from sucrose, are not known but their synthesis is essentially irreversible so $\Delta G^{\circ'}$, like that for synthesis of L. mesenteroides dextrans, is probably greater than 16 kJ.mol^{-1} (Hehre, 1946 and 1961).

Fig. 1.4 POSSIBLE UNIT STRUCTURE OF 'CARIOGENAN' MOLECULE



The glucosyltransferases which synthesise L. mesenteroides dextrans have been studied more extensively than any other similar glucosyltransferase systems. These enzymes have K_m for sucrose of 19-20 mmol/l (Hehre, 1946), and optimum activity at pH 5.0 and 30°C (Tsuchiya et al., 1952). They are inducible by sucrose (Sidebotham, 1974). The enzyme-catalysed reaction is irreversible (Hehre, 1946) and is stimulated by divalent cations (Itaya & Yamamoto, 1975). Until recently the mechanism of dextran branching was uncertain. Bailey et al. (1957) and Bovey (1959) postulated 'branching enzymes'. Bailey and co-workers found that, after storage, dextran-sucrase preparations synthesised dextrans with progressively fewer α -1,3 linkages. They suggested that a more labile branching enzyme was being inactivated during storage. Although plausible, this is obviously not conclusive. Bovey (1959) showed that the molecular weights of dextrans, as measured by periodate oxidation and light scattering, continued to increase, even after sucrose was exhausted from the incubation medium. He proposed that linear 1,6- α -D-glucan molecules were linked by enzyme action, giving higher molecular weight, branched dextran molecules. However, Ebert et al. (1966) showed that the same findings could be explained by association of low molecular weight dextran molecules to form non-covalently bound high molecular weight particles. Hehre (1969) suggested that enzyme-catalysed branching, by rearrangement of existing glucan chains (as with glycogen branching) or by formation of glycosidic links between different chains, would be energetically unfavourable, having no obvious high-energy substrate to 'drive' the reaction. However, Robyt and Taniguchi (1976) found that 'dextransucrase' preparations from L. mesenteroides could transfer [^{14}C]-labelled dextran, attached to enzyme at the reducing (C-1) end, to an unlabelled

'acceptor' dextran with formation of an α -1,3 branch linkage between the anomeric (C-1) carbon of the labelled dextran and the C-3 carbon of the acceptor dextran. The same phenomenon may also occur with the glucosyltransferase systems of S. sanguis and S. mutans. Robyt and Taniguchi (1976) argued from this that branching is a normal side-reaction of 'dextransucrases' and that it should not be necessary to postulate separate branching enzymes. However, in the absence of direct evidence that the 'dextransucrase' preparations contain only one protein species, the possibility remains that there are (at least) two enzymes, one synthesising glucan chains and the other producing branches. The possibility that this or other systems might also produce elongated branch chains by direct glucosyl transfer from sucrose appears to be eliminated by the findings that the glucans of L. mesenteroides and S. mutans are elongated by glucosyl transfer to the reducing (C-1) end of growing chains (Robyt et al., 1974; Robyt and Corrigan, 1976; discussed below).

The direction of chain growth of dextran and dextran-like glucans was until recently, controversial. Ebert and Schenk (1968a & b) first proposed that L. mesenteroides dextrans were elongated by addition of glucose from sucrose to the C-1 carbon of the reducing end of the molecule. This is unusual for glucan-synthesising glucosyltransferases. Hehre (1969) studied the action of amylosucrase, a Neisseria perflava enzyme which catalyses synthesis of an α -1,4 linked glycogen-like glucan from sucrose. He found, by pulse-labelling, that amylosucrase added glucose, from sucrose, to the non-reducing ends of preformed glycogen molecules and concluded that other glucosyltransferases, such as dextransucrase, probably acted in the same way. The growth of dextran chains by dextransucrase preparations from L. mesenteroides has been studied by

Roby et al. (1974). They used $[C^{14}]$ -sucrose pulse and pulse-chase labelling and found, by enzymic and acid hydrolysis, that C^{14} was released from the reducing end of pulse-labelled dextran but not pulse-chase labelled dextran. This indicated that newly-incorporated glucose residues are added to the reducing end of growing dextran chains. Roby et al. (1974) proposed that the dextran chains grew by an insertion mechanism rather than by simple stepwise addition of glucose to the end of the chain.

Walker (1972) also showed that α -1,3 linkages were not synthesised until well after synthesis of α -1,6 chains was initiated, since α -1,3 linkages did not appear until the nascent isomaltodextrins were at least six glucose units long.

The extracellular glucosyltransferases of oral streptococci have received more attention in recent years, starting with the work of Wood (1967b) on S. mutans and of Cybulska and Pakula (1963a, 1963b) on S. sanguis. Comparison of studies is complicated by the variety of techniques used. The various groups of workers have used many different strains of bacteria, in different culture media, grown with or without glucose or sucrose, with or without pH control and at different temperatures. Enzymes have been harvested at various ages of culture, from 6 h (Fukui et al., 1974) to 36 h (Long, 1971). The work of Beeley and Black (1977) on S. sanguis, showed that the nature of the glucosyltransferase activity is greatly affected by the age of cultures. After harvesting, the glucosyltransferases have been studied at various degrees of purification, with different yields of enzyme activity from these purifications. The structures of the glucans produced are affected by the degree of purification of the glucosyltransferases.

Highly purified glucosyltransferase preparations, from both S. sanguis and S. mutans, synthesise glucans with larger proportions of α -1,3 linkages than do crude preparations (Ceska et al., 1972; Nisizawa et al., 1977).

The results of several studies are summarised in Table 1.1 (S. mutans) and Table 1.2 (S. sanguis). In spite of the variety of experimental techniques outlined above, the findings are reasonably similar. However, many workers regard the glucosyltransferases as a single enzyme (often called 'dextransucrase'). Using isoelectric focusing (IEF), Guggenheim and Newbrun (1969) and Bulkacz and Hill (1977) showed that S. mutans produced several glucosyltransferases. The latter also showed that the multiple bands were not an artifact of IEF. However, the 13 bands which they resolved may not all be different gene products. Post-translational modifications (such as deamidation, glycosylation or subsequent deglycosylation) may affect the properties of the enzymes; such differences, unresolved by other techniques, may often be resolved by IEF. For example, some of the 'isozymes' of human α -amylase, detected by IEF or electrophoresis have been shown to be identical polypeptides modified after translation (Kauffman et al., 1970; Keller et al., 1971; Karn et al., 1974). The glucosyltransferases of S. mutans appear to be glycoproteins (Martin & Cole, 1977) and so may be subject to post-translational modification. Martin and Cole (1977) also showed that the glucosyltransferases of S. mutans could be resolved as six bands of protein by SDS-polyacrylamide gel electrophoresis. At least two, and possibly more, glucosyltransferases of S. sanguis 804 could be resolved by IEF (Newbrun, 1971) and by SDS-polyacrylamide gel electrophoresis (Dart et al., 1974). Preliminary work by Black (1975) showed that the glucosyltransferases of S. sanguis were eluted

TABLE 1.1 PROPERTIES OF GLUCOSYLTRANSFERASES OF S. mutans

STRAIN	Km (sucrose) (mmol/l)	pI	pH OPTIMUM	T OPTIMUM (°C)	Mol. Wt.	AUTHORS
FA-1	(max activity at 660)	-	6.0	-	-	Wood (1967b)
HS	7.23 0.98 3.37 4.40	4.24 5.00 5.65 6.03	6.0 7.0 6.0 7.0	-	-	Guggenheim and Newbrun (1969)
OMZ 176		4.24 4.58 5.00 5.65 6.03	-	-	-	
FA-1	-	-	6.4(6.0-6.6)	60	70,000	Long (1971)
HS-6	2.0	-	5.75	-	170,000	Fukui <u>et al.</u> (1974)
FA-1	55	3.7 4.6	6.0	-	-	Scales <u>et al.</u> (1975)
GS-5	5.6(cell-free) 8.0(cell-bound)	-	6	40	-	Kuramitsu (1974)
6715	3	4.0	5.5(4.2-6.0)	34-42	94,000	Chludzinski <u>et al.</u> (1974)
BHT	3.3	-	5.8	-	-	Balliet & Chang (1974)
6715	-	13 IEF bands from 4.56 to 7.19 4.6				Bulkacz and Hill (1977)
						Aksnes (1977)

TABLE 1.2

PROPERTIES OF GLUCOSYLTRANSFERASES OF S. sanguis

STRAIN	Km (sucrose) (mmol/l)	pI	pH OPTIMUM	T OPTIMUM (°C)	Mol.Wt.	AUTHORS
—						
Type I/II	19	—	6.3-7.0	37-45	—	Cybulska and Pakula (1963)
804	4.8	7.9	5.2-7.0	45	—	Carlsson <u>et al.</u> (1969)
804	—	7.9 6.4 4.5(minor)	5.4-7.7 5.4-7.7	—	—	Newbrun (1971)
804	2.00-2.05	—	—	—	—	Sharma and Newbrun (1973)
OMZ 9	2(cell-free) 3(cell-bound)	— } 3.8	5.8-6.0	35-40	100,000 (both)	Klein <u>et al.</u> (1976)
804	—	7.9 3.8	—	—	— *	Dart <u>et al.</u> (1974)
804	—	—	5.1-7.5	—	—	Black (1975)

*Not stated, but there were six
subunits of mol.wts. 24000-135000

from hydroxylapatite as at least four peaks of enzyme activity. As seen in Tables 1.1 and 1.2, the glucosyltransferases of S. mutans and S. sanguis have broad pH optima around pH 6. The width of the pH optimum curves in itself suggests that there may be several enzymes. It is only reasonable to suppose that there should be more than one type of glucosyltransferase present, since at least two types of glucan chain (the 'dextran' and 'mutan' types), as well as the branching linkages, are synthesised. Schachtele et al. (1977) isolated three glucosyltransferases from S. mutans. One synthesised a water-soluble glucan and may be dextranucrase-like. The other two synthesised water-insoluble glucans and may be 'mutansucrases'. Beeley and Black (1977) found that batch cultures of S. sanguis 804, at constant pH, went through three phases of glucosyltransferase production. Enzyme preparations from each phase produced chemically and biochemically distinct glucans. The findings from the different techniques used to analyse these glucans were not all mutually consistent. However, the results did indicate that enzymes with different activities were produced, and in different proportions in each phase.

Divalent cations (Ca^{2+} and Mg^{2+}) do not affect the extracellular glucosyltransferases of S. sanguis (Cybulska & Pakula, 1936b; Carlsson et al., 1969) or S. mutans (Chludzinski et al., 1974; Scales et al., 1975).

Apart from sucrose, various sugars, such as raffinose [α -D-gal-1, 6- α -D-glc-1, 2- β -D-fru], are fructosyl donors for levansucrases (e.g. that of Aerobacter levanicum). However, the dextranucrases of L. mesenteroides can use only sucrose as glucosyl donor (Hehre, 1946; Eisenberg & Hestrin, 1963). Similarly, the glucosyltransferases of S. sanguis 804 can only use sucrose; maltose, lactose, fructose, melibiose

(α -D-gal-1,6- α -D-glc), galactose, glucose, raffinose, trehalose (D-glc-1,1-D-glc), cellobiose (β -D-glc-1,4- β -D-glc) and melezitose (α -D-glc-1,3- β -D-fru-2,1- α -D-glc) produced no polysaccharide in the presence of S. sanguis 804 glucosyltransferases (Carlsson et al., 1969). With the same enzyme preparations, no glucose or fructose was released with galactose, α -methyl glucoside, lactose, maltose, trehalose, melibiose, cellobiose, melezitose or raffinose (Newbrun & Carlsson, 1969). As well as sucrose, the glucosyltransferases of S. mutans can also use leucrose (α -D-glc-1,5-D-fru), palatinose (isomaltulose; α -D-glc-1,6-D-fru), lactulosucrose (β -D-gal-1,4- β -D-fru-2,1- α -D-glc), α -glucosyl fluoride and raffinose as glucosyl donors (Taufel & Taufel, 1970; Hehre & Suzuki, 1966; Genghof & Hehre, 1972; Balliet & Chang, 1974).

Studies on potential inhibitors or glucosyl acceptors for the glucosyltransferases are affected by the method used to assay enzyme activity, measuring either glucan synthesis or release of fructose. For example, maltose increases the rate of release of fructose by S. sanguis glucosyltransferases (Newbrun & Carlsson, 1969; Klein et al., 1976) but decreases the rate of glucan synthesis by both cell-free (Newbrun et al., 1975) and cell-bound enzyme (Sharma et al., 1974). This is because, with low molecular weight acceptors such as maltose and maltotriose, the immediate reaction products are oligosaccharides (Walker, 1972; Newbrun et al., 1974). A similar effect is seen with S. mutans (Knuutila & Makinen, 1972; Chludzinski et al., 1976).

Roby and Walseth (1978) have extended the explanation of the effect of maltose. They found that glucose, fructose and maltose were acceptors for L. mesenteroides dextransucrase, giving, respectively, isomaltose, leucrose and panose (6 - α -D-glucopyranosylmaltose). Glucose

and maltose also produced the corresponding oligosaccharide series (isomaltodextrins and isomaltodextrinylmaltoses) by transfer of oligosaccharides to the acceptor molecule. At high concentrations, the acceptor molecules continuously displaced the bound glucosyl residues from the enzyme active sites, preventing their transfer to any nascent dextran chain. Thus, these acceptors acted as chain terminators rather than slow primers of glucan synthesis as was previously supposed.

Maltose and raffinose are competitive inhibitors of the glucosyltransferases of S. sanguis 804 (Newbrun et al., 1974). Although raffinose will not act as a substrate (glucosyl donor) for the glucosyltransferases of S. sanguis (Carlsson et al., 1969; Newbrun & Carlsson, 1969), its structure (a galactosylsucrose) probably sufficiently resembles that of sucrose for raffinose molecules to bind to the active sites of the enzymes.

The glucosyltransferases of S. sanguis are inhibited non-competitively by melezitose and stachyose (manneotetrose; α -D-gal-1, 6- α -D-gal-1, 6- α -D-glc-1, 2-D-fru), both sucrose derivatives. They are inhibited 'uncompetitively' by glucose and fructose (Newbrun et al., 1974; Chludzinski et al., 1976). Lactose and turanose (α -D-glc-1, 3-D-fru) have no detectable effect (Newbrun et al., 1974).

Dextran is an efficient glucosyl acceptor and stimulates the glucosyltransferases of S. sanguis (Newbrun & Carlsson, 1969) and S. mutans (Fukui et al., 1974) by acting as a primer for chain elongation. Robyt and Corrigan (1977) claim that stimulation of glucosyltransferases is not by a primer mechanism. However, their study merely showed that new glucosyl residues were not added to the non-reducing end of dextran molecules. This should have not been expected anyway, in view of their

earlier demonstration (Robyt et al., 1974) that L. mesenteroides dextran chains grew by addition to the reducing end of the molecules. Given that this is correct, it is reasonable that the reducing ends of dextran molecules should act as primers since that is how the glucan chains are elongated, even in the absence of added acceptors.

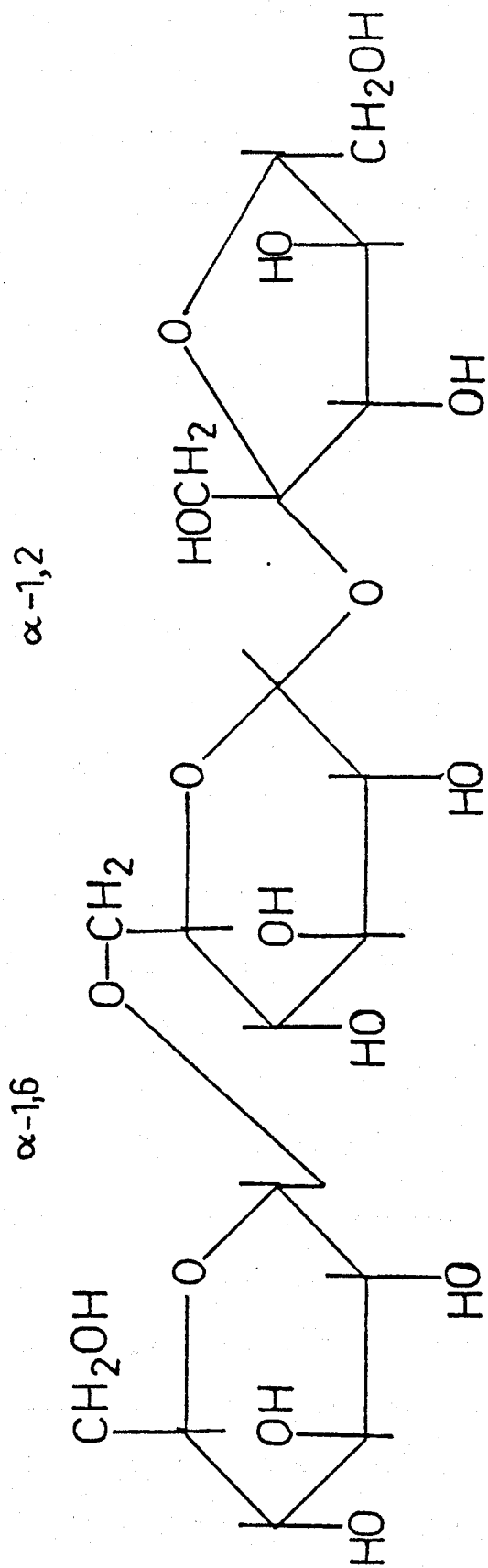
Klein et al. (1976) reported that the apparent K_m (K_m^{app}), for sucrose, of the glucosyltransferases of S. sanguis, was not affected by the concentration of 'primer' dextran when activity was measured as release of fructose. However, when the activity of S. mutans glucosyltransferases was measured as synthesis of precipitable glucan, K_m^{app} was decreased by added dextran (Chludzinski et al., 1976). Although the Lineweaver-Burk plots for the S. sanguis glucosyltransferases did not show the complex non-Michaelis-Menten kinetics of the S. mutans enzymes, one would not expect the two systems to behave very differently. However, the apparent discrepancy between the two sets of findings can be explained. In the absence of added dextran (or other acceptors), sucrose is the predominant glucosyl acceptor. As an acceptor, initiating chain synthesis, sucrose will lead mainly to production of oligosaccharides, initially at least; some time will be required for elongation to high molecular weight glucans, as shown by Chludzinski et al. (1976). Thus, K_m^{app} for glucan synthesis will be high. In the presence of added dextran, a higher proportion of enzyme-substrate complexes will produce precipitable glucan since the acceptor is already a high molecular weight glucan; thus, K_m^{app} , for glucan synthesis, will be reduced. Fructose is released with glucosyl transfers from sucrose, whatever the acceptor or reaction product. Therefore, K_m^{app} for fructose release should be little affected by the nature and concentration of glucosyl acceptors present.

Although dextran stimulates synthesis of glucans, it has been reported that low molecular weight dextrans inhibited synthesis of insoluble glucans by S. mutans in batch culture and by S. mutans glucosyltransferases (Gibbons & Keyes, 1969; Montville et al., 1977). The former only measured glucan synthesis as increase in turbidity at 350 nm, of incubation mixtures. Montville and co-workers showed that total glucosyltransferase activity was, in fact, stimulated by low molecular weight dextran. Therefore, these dextrans may be efficient acceptors for synthesis of soluble, dextran-like glucans, but not insoluble mutan-like glucans or more complex high molecular weight glucans. Moreover, Shaw (1972) found that this effect, which was only demonstrated in vitro, was not able to reduce the incidence of dental caries in laboratory animals.

Isomaltose and isomaltotriose are glucosyl acceptors for the glucosyltransferases of S. sanguis and S. mutans (Walker, 1972; Fukui et al., 1974). Germaine et al. (1974) reported that isomaltose did not affect the activity of the S. mutans enzymes but used an isomaltose concentration of only 20 $\mu\text{mol/l}$. Walker used 35 mmol/l . (Fukui and his co-workers did not specify what concentration they used.)

Trehalose stimulates the activity of S. sanguis glucosyltransferases (Klein et al., 1976). Theanderose ($6^G\text{-}\alpha\text{-D-glucopyranosylsucrose}$, see fig. 1.5) stimulates the glucosyltransferases of Streptococcus type 6, an S. sanguis-like strain, and is apparently a more efficient glucosyl acceptor than sucrose (Walker, 1972). In both of these sugars, the anomeric carbon atoms are all involved in glycosidic linkages and it was formerly believed that glucan chains would grow from these acceptors by glucosyl transfer to the non-reducing (C-6) end of the molecules. In the mechanism described for L. mesenteroides dextransucrase by Robyt

Fig. 1.5 THEANDEROSE



et al. (1974), glucosyl residues transferred from sucrose are bound to either one of two sites as glucosyl-enzyme complexes before they are transferred to the nascent glucan or isomaltodextrin chain. With trehalose, whose free energy of hydrolysis is probably only slightly less than that of sucrose (Cabib & Leloir, 1958), the same mechanism should be feasible. With theanderoose, the glucose-fructose linkage might be hydrolysed, just as with sucrose, but an isomaltosyl group would be transferred to the enzyme. A glucosyl residue, covalently bound to the other site on the enzyme, would be transferred to the isomaltosyl group; this is perfectly compatible with Robyt's mechanism. Another interesting possibility, for which there is yet no evidence, is that the 'glucosyltransferases', using theanderoose as substrate, might also transfer one isomaltosyl group to another by a similar mechanism to the one just described but, possibly, with double the rate of chain growth.

Glycogen, amylose, inulin and raffinose do not stimulate the glucosyltransferases of S. mutans, with sucrose as substrate (Germaine et al., 1974; Chludzinski et al., 1976). Similarly, raffinose and 'levan' (S. salivarius fructan) do not stimulate the glucosyltransferases of S. sanguis (Newbrun & Carlsson, 1969).

As described in the preceding paragraphs, there have been many enzymological studies of the streptococcal glucosyltransferases. However, the lack of sufficiently purified or well characterised preparations has precluded any detailed molecular studies, such as amino acid sequencing or studies of the molecular structure of the active sites of the enzymes. The S. mutans glucosyltransferases are probably glycoproteins, having tightly bound carbohydrate moieties (Germaine et al., 1974; Martin & Cole, 1977). Some of the glucosyltransferases, at least, contain

several sub-units. The enzyme activities of the S. sanguis and S. mutans glucosyltransferases are destroyed by sodium dodecyl sulphate (SDS) (Dart et al., 1974; Chludzinski et al., 1974) or by other detergents (Jablonski & Hayashi, 1970). Dart et al. (1974) separated the purified glucosyltransferases of S. sanguis 804, by SDS-polyacrylamide gel electrophoresis, into six 'sub-units'. Each 'sub-unit' individually lacked enzyme activity and they had molecular weights of about 135,000, 92,500, 75,000, 47,500, 31,500 and 24,500. The values of these molecular weights are, however, also consistent with the first, third and fifth 'sub-units' representing the tetramer, dimer and monomer of one polypeptide, and the second, fourth and sixth representing another.

Some sulphydryl reagents inhibit the glucosyltransferases of S. mutans (Chludzinski et al., 1974). The S. sanguis enzymes are not affected (or are more resistant) (Carlsson et al., 1969).

Using highly purified (770-fold) glucosyltransferases of S. sanguis 804, Callaham and Heitz (1973) showed that, in visible light, the enzymes were inactivated by rose bengal, a compound which stimulates photo-oxidation of histidine and tryptophan. The enzymes were protected from this inactivation by pre-incubation with dextran, suggesting that the point of attack by rose bengal is at or near the binding site for glucosyl acceptors. In the same study, eosin (yellowish), which is similar to rose bengal, was a reversible, competitive inhibitor with K_i (inhibitor constant) of 379 $\mu\text{mol/l}$. The effect of rose bengal was greater at low pH, where histidine is resistant to this photo-oxidation effect (Callaham & Heitz, 1974). N-bromosuccinimide and hydroxy-5-nitrobenzyl bromide attack tryptophan residues (although the latter is not specific in this) and both inactivated the glucosyltransferases. N-bromosuccinimide and rose bengal both decreased the normal fluorescence

of tryptophan (Callaham & Heitz, 1974). Unfortunately, there have been no other studies on the active sites of these glucosyltransferases. However, the data just described do suggest that there is at least one tryptophan residue at the active site, essential for enzyme activity, and that the substrate (donor and acceptor) binding sites are near this residue.

1.4 AIMS OF THE STUDY

Beeley and Black (1977) and Black (1975) studied the production of extracellular glucosyltransferases by S. sanguis 804 in batch culture. The original aim of this study was to continue this work and to purify and characterise these enzymes. S. sanguis was used for various reasons. It is one of the first bacteria to bind to the tooth surface in plaque formation (see Sections 1.2.3 and 1.2.4). Invertase, which might complicate studies of glucosyltransferase activity, is constitutive in S. mutans but inducible by sucrose in S. sanguis (McCabe et al., 1973). The presence of invertase would preclude the use of glucosyltransferase assays based on measurements of release of fructose. Even with assays completely specific for glucosyltransferase activity, hydrolysis of sucrose by invertase might complicate any kinetic studies of the glucosyltransferases. By studying S. sanguis, grown in the presence of glucose, these problems can be avoided.

Beeley and Black (1977) showed that production of glucosyltransferase by S. sanguis 804, in batch culture, is triphasic. Phase II of glucosyltransferase production was selected for study, for two reasons. Of the three phases, phase II is the most easily identified; it occurs at the end of the logarithmic growth phase, when the culture medium is depleted of glucose. Moreover, this phase seems to correspond closely to that studied by most other workers. This facilitates comparison of the results from this study with those obtained by other workers.

2 : MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

Dextrans T10 and T2000 were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Kojibiose was obtained from Koch-Light Laboratories Ltd., Colnbrook, England. Nigerodextrins, from nigerose to nigeropentaose, prepared by hydrolysis of Aspergillus niger glucan (nigeran), were a gift from Dr. I.R. Johnston, Dept. of Biochemistry, University College, London.

Metrizamide [2-(3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose] was a gift from Nyegaard and Co., AS, Oslo, Norway.

All other reagents were of analytical reagent grade, except sucrose for glucosyltransferase assays which was 'Aristar' grade (BDH Chemicals Ltd., Poole Dorset, England).

2.1.2 Culture Media

(i) Blood Agar

40 g Blood Agar Base (Oxoid Ltd., Basingstoke, England) was dissolved in 1 l distilled water, steamed over boiling water for 20 min and then autoclaved for 20 min at 103.5 kPa. The agar was cooled to 43°C and defibrinated horse blood (Wellcome Research Laboratories, Beckenham, Kent) was added to a final concentration of 5% (v/v).

(ii) Carlsson's Diffusate Medium

This medium, described by Carlsson et al. (1969), was made up

as follows. 25 g Casein Hydrolysate (Acid), 100 g Tryptose and 50 g Yeast Extract (all from Oxoid Ltd.) were dissolved in 500 ml distilled water and dialysed in Visking tubing against 1.5 l distilled water at 4°C, overnight. The dialysis was repeated twice more and the three diffusates were pooled. The volume was made up to 4 l with distilled water and 15 g of anhydrous K_2HPO_4 was added and dissolved. This solution was autoclaved at 103.5 kPa for 20 min (or longer, depending on the volume of liquid to be sterilised). If the medium was not to be autoclaved immediately it was stored frozen at -20°C.

50 g/l (277 mmol/l) D-glucose, in distilled water, was autoclaved at 103.5 kPa for 20 min and added aseptically to sterile Carlsson's Diffusate, as described above, to give a final glucose concentration of 10 g/l (55.5 mmol/l).

2.2. MICROORGANISMS

Streptococcus sanguis 804 (NCTC 10904), a strain isolated from human dental plaque, was a gift from Prof. J. Carlsson, University of Umeå, Uppsala, Sweden. Bacteria were stored as freeze-dried cultures or as sub-cultures on blood agar (see 2.1.2) slopes grown overnight at 37°C and stored at 4°C with fortnightly transfer.

2.3 METHODS

2.3.1 Growth of *S. sanguis* 804

S. sanguis 804 was grown in batch culture as described by Beeley and Black (1977). Various volumes of culture medium, all in conical flasks and from 0.25 to 4 l, were used.

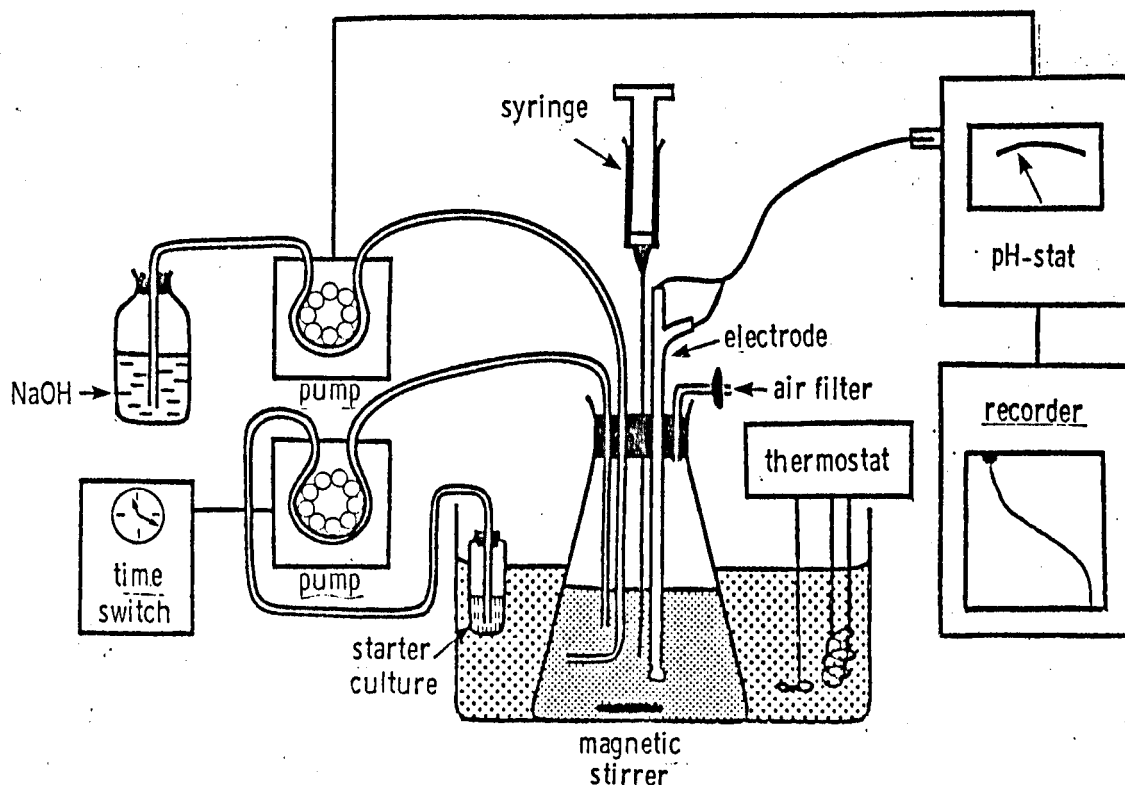
Bacteria were grown in Carlsson's Diffusate Medium (see 2.1.2) containing 10 g/l D-glucose, at 37°C and at a constant pH of 7.0 ± 0.1 , in the apparatus shown in fig. 2.1.

Cultures were inoculated with a 16 h liquid starter culture in the same medium, 2% of the culture volume, using a peristaltic pump and an automatic time switch. pH was controlled by means of a steam-autoclavable combination glass electrode (Activion Glass Ltd., Leslie, Fife, Scotland) and a Radiometer Titrator TT1 (Radiometer A/S, Copenhagen, Denmark) which regulated addition of 1.0 mol/l NaOH (10 mol/l for 4 l cultures) by a peristaltic pump.

Five different parameters were monitored during culture growth.

- (1) Depletion of glucose from culture fluid was measured quantitatively by the 'God-Perid' method (see section 2.3.3) and semi-quantitatively, for rapid estimations, using 'Clinistix' reagent strips (Ames Co., Slough, England).
- (2) Cell number was measured as turbidity (E_{540}) of culture fluid. Black (1975) has shown for these cultures that turbidity of culture fluid, at 540 nm, is directly proportional to dry weight of washed cells, up to a turbidity of 1.4 (equivalent to about 250 µg dry weight of cells/ml). Samples of high cell density ($E_{540} > 0.8$) were diluted 1:10 in 0.9% w/v (154 mmol/l) NaCl before turbidity was measured.

Fig. 2.1 BATCH CULTURE APPARATUS



NOTES

- (i) The temperature of the culture vessel was maintained at 37°C in a thermostatically controlled circulating water bath.
- (ii) The pH of the culture was kept constant at 7.0 ± 0.1 by addition of NaOH, regulated by a pH-stat, as described in the text (p.46).
- (iii) The air in the culture was kept sterile by means of a glass fibre or sintered glass filter.
- (iv) Sterility of the culture was checked by examining Gram stains and by plating out samples on blood agar (see 2.1.2) at regular intervals.

- (3) Acid production was monitored by a chart recorder (Radiometer Titrigraph SBR1c) connected to the microswitch relay which controlled addition of NaOH. The chart trace was a measure of cumulative pumping time of NaOH solution. The total amount of acid produced by the culture, at any given time, could be calculated from this trace, knowing the flow rate (pumping rate) and the concentration of the NaOH.
- (4) Glucosyltransferase activity in culture supernatants was measured by the method of Cybulska and Pakula (1963a), as described in section 2.3.3.
- (5) Protein concentration in culture supernatants was measured by the method of Lowry et al. (1951) as described in section 2.3.3.

2.3.2 Treatment of culture fluid

Before the concentrations of glucose and protein were assayed, samples were chilled on wet crushed ice and bacteria were removed by centrifugation at 1600g for 20 min at 4°C in an MSE '4L' centrifuge. The essentially cell-free supernatant will be referred to, in this thesis, as 'culture supernatant'.

2.3.3 Assay methods

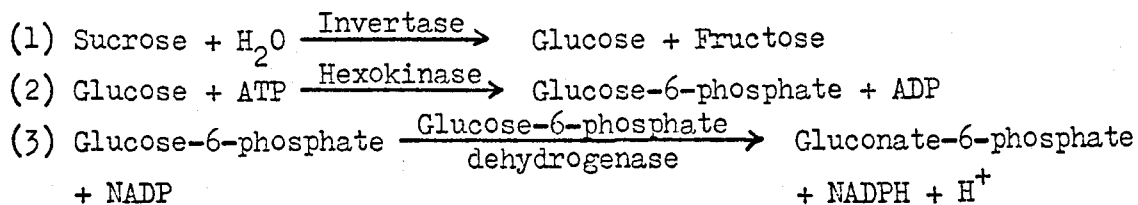
(i) Glucose

The glucose concentration in culture supernatants was determined by the glucose oxidase-Perid method using Blood Sugar test kits (Boehringer Mannheim, GmbH, Mannheim, West Germany).

(ii) Sucrose/Glucose

Sucrose and glucose concentrations in saliva, foods and drinks, and in the extracellular fluid of dental plaque were assayed by an

enzyme-coupled system using Sucrose/Glucose UV test kits (Boehringer Mannheim GmbH, Mannheim, West Germany) with the following reaction scheme:



Production of NADPH was measured as increase in E_{340} . In the absence of invertase, ΔE_{340} is proportional to the concentration of glucose in the sample. In the presence of invertase, sucrose is broken down to glucose and fructose, therefore ΔE_{340} is proportional to the total concentration of sucrose and glucose. Thus, the concentration of sucrose is proportional to the difference between the values of ΔE_{340} in the presence and the absence of invertase.

(iii) Protein

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine α -chymotrypsinogen as a standard (Wilcox et al., 1957).

0.4 ml of sample (at neutral pH) was added to 0.6 ml of 1.0 mol/l NaOH. 5 ml of copper citrate in sodium carbonate was added; the solution was then mixed and incubated at room temperature for 10 min. 0.5 ml of a 1:2 dilution of the Folin-Ciocalteu reagent (in H_2O) was added, mixed rapidly and incubated at room temperature for 30 min. Extinctions were then read, immediately, at 500 nm.

The copper citrate-sodium carbonate reagent was prepared by diluting 1 volume of solution of 0.5% w/v $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1.0% w/v sodium citrate with 49 volumes of a 2% w/v solution of Na_2CO_3 .

Protein concentrations in column eluates were monitored as E_{280} . No attempt to standardise these measurements was made.

(iv) Carbohydrate

The carbohydrate concentration in glucan preparations was estimated as total hexose by the method of Dubois et al. (1956), using glucose as a standard, as follows. 1 volume of 5% w/v (531 mmol/l) aqueous phenol was added to 1 volume of sample in a thick-walled 'Pyrex' test-tube. Five volumes of 96% (v/v) H_2SO_4 were added and mixed rapidly. The solution was left to stand at room temperature for 10 min and was then cooled to 25°C in a circulating water bath for 20 min. Extinctions were measured at 490 nm; if the tubes are covered (to prevent dust settling on the contents) the colour is stable for over 24 h.

(v) Glucosyltransferase activity

Enzyme activity was measured by two methods.

Precipitable glucan production

For crude preparations and in some experiments, the method of Cybulska and Pakula (1963a), as modified by Beeley and Black (1977) was used.

One volume of enzyme preparation was mixed with one volume of 10% (w/v) 'Aristar' sucrose (292 mmol/l) in 100 mmol/l pH 7.0 sodium phosphate buffer containing 0.2 g/l penicillin/streptomycin (tissue culture preparation; BDH Chemicals Ltd., Poole, Dorset, England). The mixture was incubated at 37°C for 24 h (or less, as the experiment demanded). Reactions were stopped by adding 2 volumes of 10% (w/v) (1.22 mol/l) sodium acetate followed by 5 volumes of 96% ethanol at 4°C. The mixture was shaken to precipitate polysaccharide and left for at least 4 h at room temperature. The precipitate was centrifuged at 1000g for 20 min at room temperature and was washed twice with a mixture of 96% ethanol, 10% (w/v) sodium acetate and distilled water (5:2:2 by

volume). The washed precipitates were redissolved in 0.1 mol/l NaOH and assayed for total hexose by the method of Dubois et al. (1956) as described above. Precipitates which were only sparingly soluble were redissolved in 1.0 mol/l NaOH (Dr. M.V. Martin, personal communication).

Control tubes were those tubes to which sodium acetate and ethanol were added at zero time.

Determination of release of fructose

This method was used for partially purified preparations of enzyme and for column eluates. (Method of Carlsson et al., 1969).

One volume of enzyme solution was incubated with one volume of 250 mmol/l sucrose in 100 mmol/l pH 6.8 sodium phosphate buffer at 37°C. At zero time and at a specified incubation time (usually 1 hour), two 0.2 ml samples were withdrawn and each was added to 0.8 ml of 40 mmol/l NaOH solution. The samples were then assayed for reducing sugar (as fructose) in alkaline conditions by the method of Somogyi (1945). The samples in NaOH were mixed with 2 ml of the Somogyi copper reagent. The mixtures were incubated at 100°C in covered test tubes for 10-20 minutes and then cooled to room temperature (20-25°C) in a water bath. Nelson's arsenomolybdate reagent, sufficient to dissolve the cuprous oxide precipitate (usually 1 ml), was added and the volume was made up to 6 ml (or 25 ml with samples of high enzyme activity). The colour produced is proportional to the concentration of fructose. Extinction (at 520 nm) may be read immediately. The colour is stable for several hours but will deteriorate overnight. For greater sensitivity, extinction may be read at 540-600 nm.

(vi) Hydrogen peroxide

Hydrogen peroxide solutions were standardised by the method of

Vogel (1951; pp.348-349). A sample of '100 volume' hydrogen peroxide (Merck 'Perhydrol') was diluted 1:5 and 10 ml of the diluted sample was placed in a conical flask. 180 ml of 2 N H_2SO_4 was added and CO_2 was bubbled slowly through the mixture. 20 ml of 10% w/v potassium iodide, followed by 3 drops of 20% w/v ammonium molybdate were then added, and the released iodine was titrated with 0.1 N $\text{Na}_2\text{S}_2\text{O}_3$.

(vii) ZrOCl_2 assay for fructose

The glucosyltransferase assay method involving assay of release of fructose from sucrose as reducing sugar in alkaline conditions (Carlsson et al., 1969) is rapid and convenient but cannot be used to assay enzyme activity in crude preparations because of interference by other compounds (Black, 1975).

In the method used by Carlsson et al. (1969), fructose was assayed by the method of Somogyi (1945). The fructose assay technique described by Schlegelová and Hruška (1977) was investigated as a possible alternative to the Somogyi method. In this procedure, 500 μl of the sample was mixed with 500 μl of 40 mmol/l zirconyl chloride (ZrOCl_2) solution. The mixture was heated at 100°C for 30 minutes, cooled in an ice bath for 2 minutes, and diluted to a final volume of 3-10 ml with distilled water. The extinction was measured at 334 nm. Ultraviolet spectra of the resulting colour were obtained with a Pye-Unicam SO 800 scanning spectrophotometer.

2.3.4 Purification of *S. sanguis* 804 glucosyltransferases

Extracellular glucosyltransferases in culture supernatants were purified by the following series of procedures:
Concentration/Desalting of culture supernatant.

Ammonium sulphate precipitation.

Desalting of ammonium sulphate precipitates.

Hydroxylapatite chromatography.

(The desalting steps were omitted in earlier preparations.)

(i) Concentration/Desalting

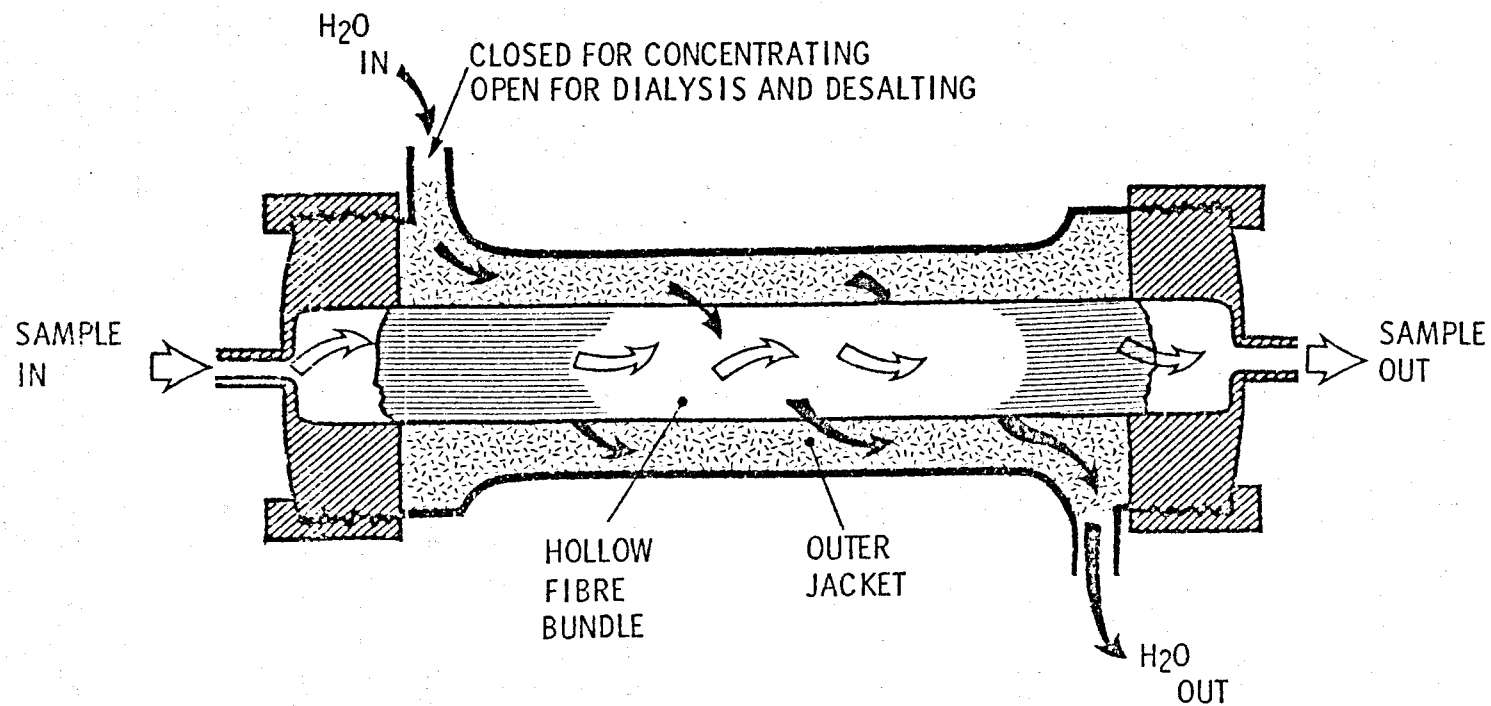
Culture supernatants were concentrated and desalted against distilled water in a Bio-Fiber 80 Miniplant hollow fibre ultrafiltration device (mol.wt. cut-off 30,000; Bio-Rad Laboratories Ltd., Bromley, Kent) in order to:

- (a) reduce large culture supernatant volumes
- (b) remove possible low molecular weight enzyme inhibitors and/or denaturing agents
- (c) remove oligopeptides to facilitate further purification.

Samples of culture supernatant were circulated around the hollow fibre bundle (fig. 2.2) by means of a peristaltic pump connected to the ports on the surrounding jacket. Water and other low molecular weight species were drawn through the pores in the fibre walls, and out of the fibres, by another peristaltic pump. Small samples (less than 1 litre) were concentrated 2-fold, and larger samples up to 4-fold. Various flow rates, of 1-20 ml/min, were used, depending on the volume of culture supernatant used.

Redissolved ammonium sulphate precipitates were desalted by dialysis against the resuspending buffer (see next section) in a Bio-Fiber 80 beaker. (This is essentially the same device as the Miniplant but has a slightly different configuration and a lower fluid capacity). The whole sample was placed inside the hollow fibres and dialysed against buffer which was circulated through the surrounding jacket at about 1 ml/min.

Fig.2.2 OPERATION OF 'BIO-FIBER 80 MINIPLANT' ULTRAFILTRATION DEVICE



All procedures were performed at 4°C in a cold room.

(ii) Ammonium sulphate precipitation

Finely powdered, dried ammonium sulphate was added slowly to concentrated culture supernatant, in an ice bath, with stirring. When the desired concentration of ammonium sulphate was reached (according to the nomogram of Dixon (1953) with correction for temperature), the mixture was kept stirring for at least 30 min and the precipitate was removed by centrifugation at 20,000 g for 25 min at 4°C.

Precipitates were dissolved in 1 mmol/l potassium phosphate buffer (pH 6.8), using the minimum volume necessary to dissolve the precipitate, and the solution was either used immediately or stored frozen at -20°C.

(iii) Hydroxylapatite chromatography

Various volumes and shapes of chromatography column were used. The details of these are stated in the results section. All experiments were performed at 4°C. In all experiments, Sigma hydroxylapatite (Type I; de-fined suspension), equilibrated with 1 mmol/l potassium phosphate buffer (pH 6.8) was used. Columns were regenerated by washing with at least 2 column volumes of 1.0 mol/l aqueous NaCl, followed by at least two column volumes of the starting buffer.

Stepwise gradient elution

Samples of glucosyltransferase solution (purified by ammonium sulphate as described above) were applied manually to a hydroxylapatite column, via a three-way tap.

Fractions were eluted by increasing concentrations of buffer

(0.001-1.0 mol/l potassium phosphate, pH 6.8) in a step-wise gradient. Eluant buffer was pumped through the column (descending elution) by means of a peristaltic pump, connected to the bottom of the column. Potassium phosphate buffer was used because, at 4°C, sodium phosphate buffers are not sufficiently soluble at the higher concentrations used (up to 1.0 mol/l).

The protein concentrations (as E_{280}) and glucosyltransferase activities of fractions were monitored.

Continuous gradient elution

The procedure was identical to that for step-wise elution except that fractions were eluted by continuous gradients programmed automatically by an 'Ultragrad' electronic gradient mixer (LKB-Produkter, AB, Sweden).

(iv) Experiments on affinity chromatography of glucosyltransferases

The glucosyltransferases of S. sanguis 804 bind strongly to Sephadex (Pharmacia Fine Chemicals; cross-linked dextran gel beads) (Carlsson et al., 1969). The use of Sephadex as a possible medium for affinity chromatography of these enzymes was therefore investigated. If practicable, this method would be preferable to using a specially prepared column material, e.g. by binding dextrans to epoxy-activated Sepharose.

Sephadex G-10 was equilibrated in 0.1 mol/l sodium phosphate buffer (pH 6.8) and all eluants were made up in the same buffer. Pharmacia K9/15 (diam. 9 mm x height 150 mm) columns were used. Column bed volumes were 9.5 ml. Column void volumes and evenness of packing of the Sephadex were determined by means of Blue Dextran 2000 (Pharmacia). Constant flow rates were maintained by peristaltic pumps (LKB Perpex) and fractions were collected manually. Stepwise elution gradients were used. Samples were eluted with glucose (0 - 1.0 mol/l) or Dextran T2000

(Pharmacia; mol.wt. average 2×10^6) (0 - 1.0 g/l, equiv. to 0 - 0.5 $\mu\text{mol/l}$). Flow rates were about 0.25 ml/min and fraction sizes were about 3.5 ml.

1.0 ml of ammonium sulphate purified glucosyltransferase preparation, containing 1.12 mg of protein and with glucosyltransferase activity of 57.1 mg precipitable glucan/24 h, was applied to each column.

2.3.5. Purification of nigerodextrins

(i) Homogeneity of preparations - paper chromatography

Samples of nigerodextrin solutions (5 μl of 2 mg/ml in water) were applied to Whatman 3MM chromatography paper and run by ascending chromatography using propan-1-ol:ethyl acetate: H_2O (6:1:3, v:v:v) as solvent.

At the end of the runs, the chromatograms were dried, sprayed with ammoniacal silver nitrate (50 mmol/l AgNO_3 , 2.5 mol/l ammonia) and heated at 105°C for 10 min. Nigerodextrins appeared as dark brown spots on a pale brown background.

Mobility of sample was measured as R_{glc} , that is, the ratio of distance moved by the spot to the distance moved by glucose.

All preparations appeared to be homogeneous, except nigerotriose and nigerotetraose which showed faint spots of lower mobility (see Table 2.1).

(ii) Purification by gel filtration on Bio-Gel P-2

Those preparations shown to be heterogeneous by paper chromatography (i.e. nigerotriose and nigerotetraose) were purified by gel filtration on Bio-Gel P-2 (100-200 mesh).

Table 2.1 PAPER CHROMATOGRAPHY OF NIGERODEXTRINS AND STANDARDS

SAMPLE	R_{glc}	
	EXPERIMENTAL	REPORTED BY JOHNSTON (1965)
Glucose	(1.00)	(1.00)
Nigerose	0.81	0.82
Nigerotriose	0.69; 0.66 (i)	0.67
Nigerotetraose	0.49	0.5
Nigeropentaose	0.34 (ii)	0.42 (ii)
Lactose	0.65	-
Maltose	0.67	-

NOTES

- (i) All results are from at least two determinations. Only these two showed any variation on different runs.
- (ii) The difference between the result found here, and that reported by Johnston, is difficult to explain. An R_{glc} of 0.34 corresponds more closely to nigerohexaose.

A column of Bio-Gel P-2, 25 mm (d) x 257 (h) (bed volume = 126 ml), equilibrated with 100 mmol/l pH 7.0 sodium phosphate buffer, was used. Samples dissolved in the buffer were applied to the column and were eluted at a flow rate of 1.0 ml/min (fraction sizes, 1.0 - 2.1 ml) using the same buffer.

The column was calibrated for molecular weight with a mixture of D-glucose, maltose, T-10 Dextran (Pharmacia) and Blue Dextran 2000 (Pharmacia).

Fractions were assayed for total hexose by the method of Dubois et al. (1956; see 2.3.3).

Blue Dextran and T-10 Dextran were eluted with the void volume (50 ml) (see fig. 2.3). Maltose was eluted at $V_e/V_o = 1.81$ and glucose at $V_e/V_o = 1.98$.

On the same column nigerotriose eluted at $V_e/V_o = 1.77$, with some minor peaks of lower molecular weight (see fig. 2.4). Nigero-tetraose was eluted at $V_e/V_o = 1.67$ as a single, symmetrical carbohydrate peak (see fig. 2.5). The minor spots observed in paper chromatography of the nigerotetraose may have been artifacts or may have been non-carbohydrate contaminants, since the silver nitrate reagent reacts also with reducing compounds other than carbohydrates.

Fractions containing the desired nigerodextrins were pooled (see figs. 2.4 and 2.5), freeze-dried and stored.

2.3.6 Pycnometry with organic solvent density gradients

(i) Introduction

To study clearance of sucrose from dental plaque and to determine

Fig.2.3 CHROMATOGRAPHY OF CARBOHYDRATE STANDARDS ON BIO-GEL P-2

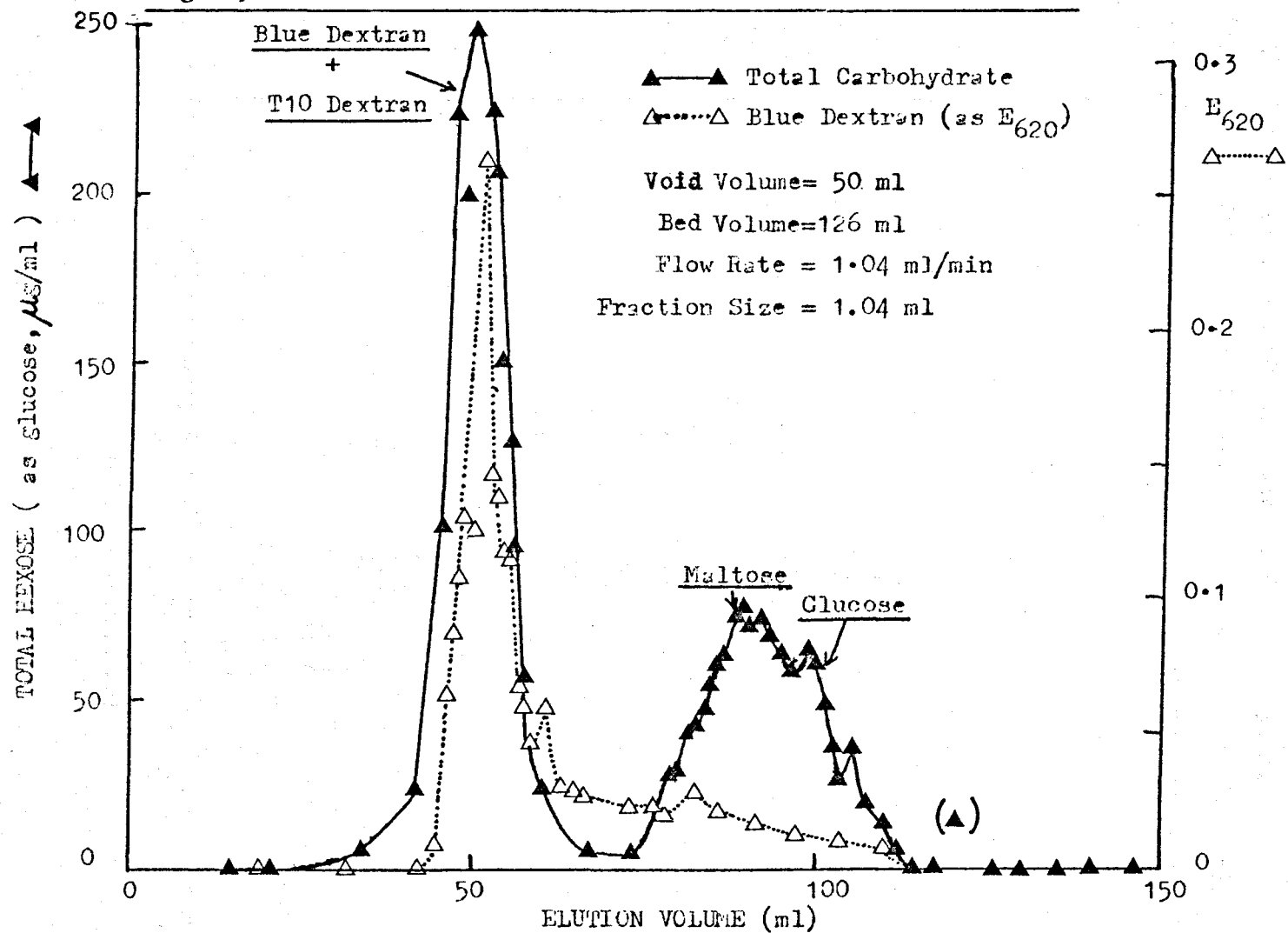


Fig 2.4 CHROMATOGRAPHY OF NIGEROTHIOSE PREPARATION ON BIO-GEL P-2

NOTES

- (i) Void Volume= 50 ml. Bed Volume= 126 ml.
- (ii) Flow Rate= 0.16 ml/min. Fraction Volume= 0.99 ml
- (iii) Sample Volume= 2.5 ml. Sample Size= 145 mg (as glucose).
- (iv) Fractions from the hatched area were pooled and freeze-dried.

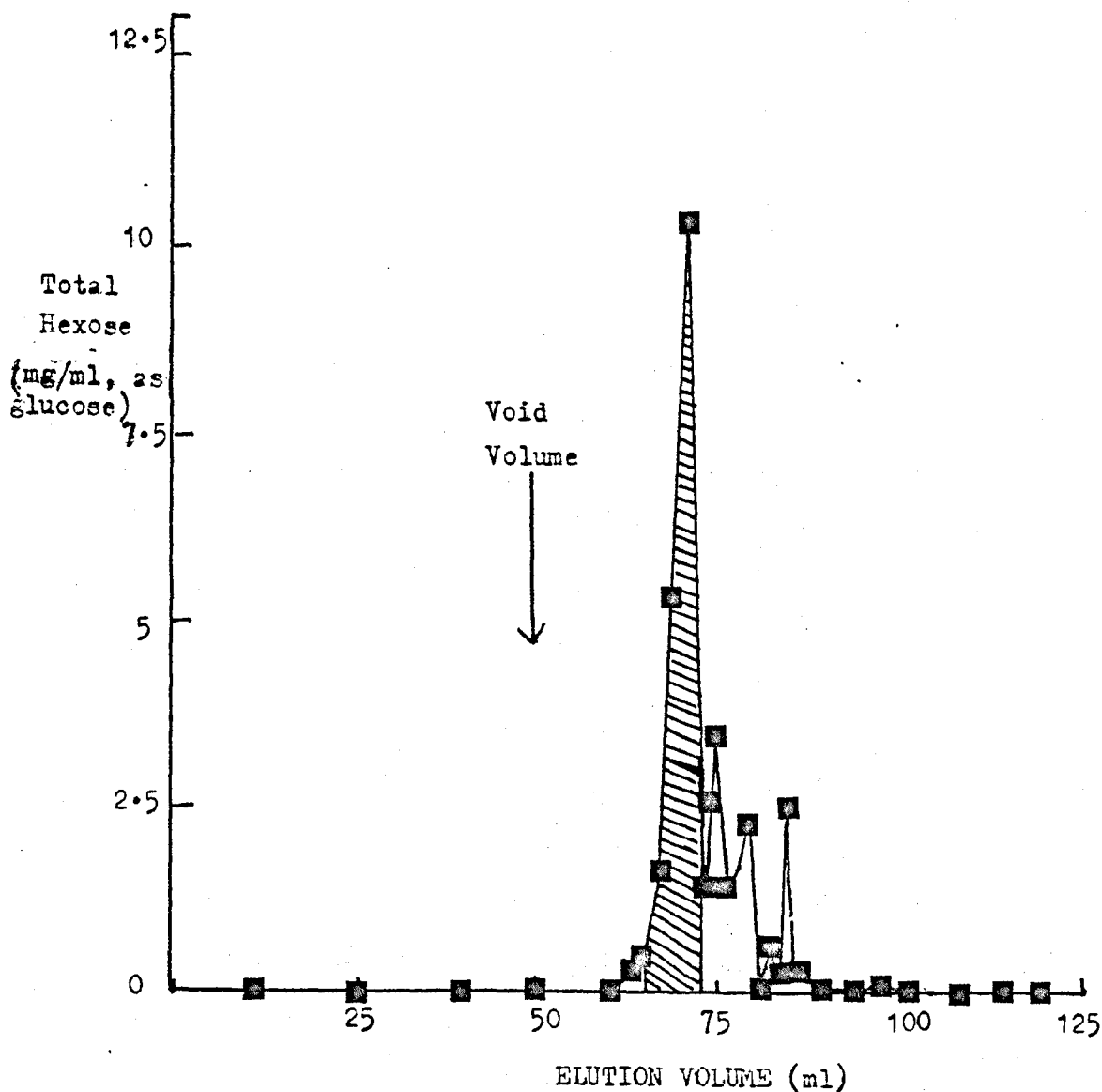
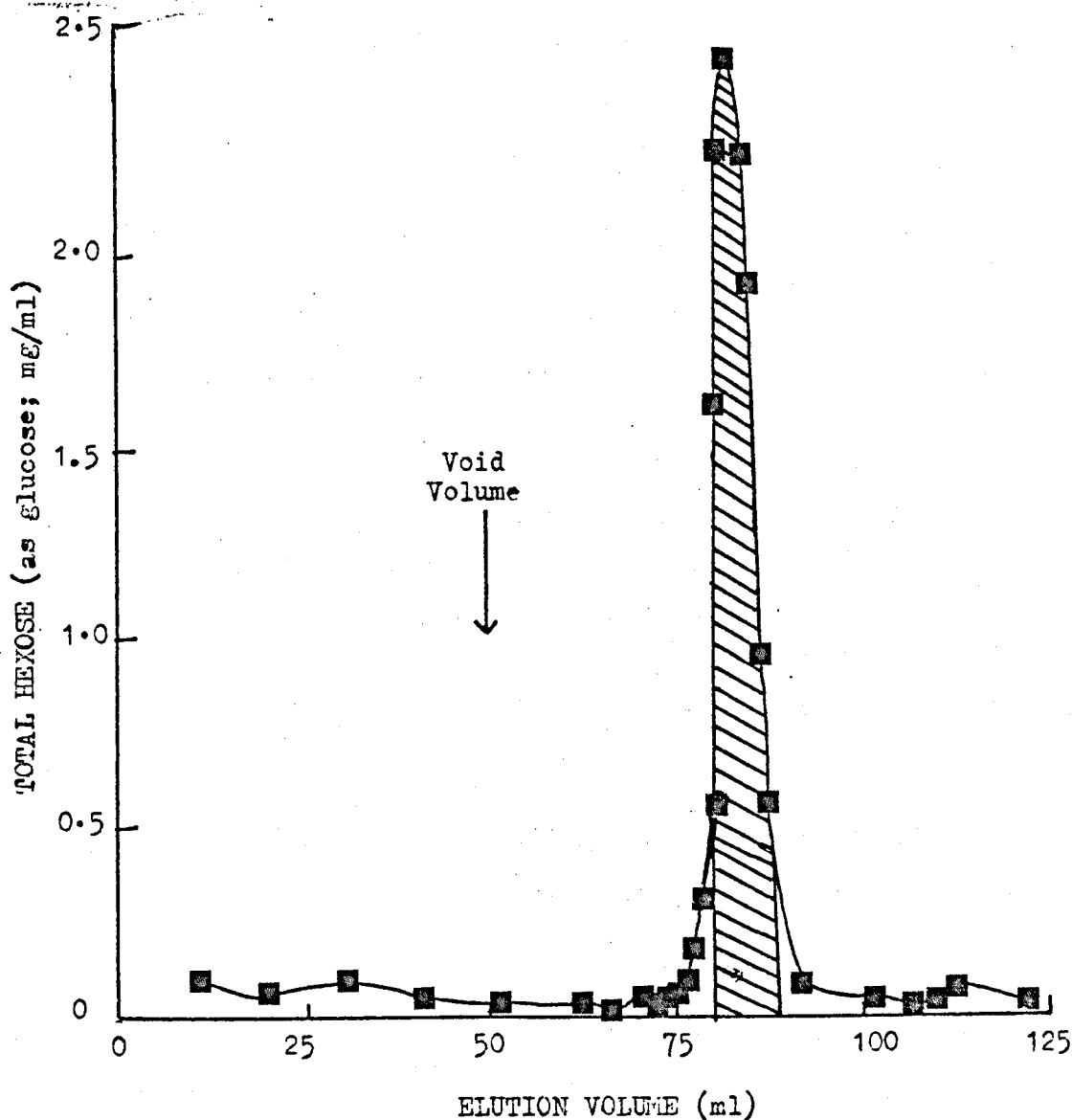


Fig. 2.5 CHROMATOGRAPHY OF NIGEROTETRAOSE PREPARATION

ON BIO-GEL P-2

NOTES

- (i) Void Volume= 50 ml. Bed Volume= 126 ml.
- (ii) Flow Rate= 0.16 ml/min. Fraction Volume= 1.02 ml.
- (iii) Sample Volume= 0.8 ml. Sample Size= 11.8 mg.
- (iv) Fractions from the hatched area were pooled and freeze-dried.



the concentrations of sucrose in plaque fluid, it is essential to know the volume of extracellular fluid (ECF) in any given plaque sample. It is not feasible to measure the volume directly since the volumes involved are so small. For example, the total plaque from an adult human mouth is typically about 20 mg; assuming about 31% ECF (Edgar and Tatevossian, 1971), this gives an ECF volume of about 6 μ l, depending on the density of the fluid.

A method of determining the density of very small samples of liquid was therefore required. Density measurement techniques using gradients of organic solvents have been in use for some time (see following paragraphs). If a drop of an aqueous liquid is applied to the top of such a gradient, the drop will sink until it reaches the point at which it has neutral buoyancy (the isopycnic point). By calibrating the gradient with drops of solution of known density one can determine the density of unknown solutions.

Gradients have been made using CCl_4 ($\rho^{20} = 1.6 \times 10^3 \text{ kg.m}^{-3}$) or bromobenzene ($\rho^{20} = 1.5 \times 10^3 \text{ kg.m}^{-3}$) as the 'heavy' solvent and petroleum spirit (petroleum ether) or odour-free kerosene (for both $\rho^{20} = 0.6\text{--}0.7 \times 10^3 \text{ kg.m}^{-3}$, depending on the hydrocarbon 'cut' or mol.wt. fraction selected) as the 'light' one. Various methods of preparing gradients have been described. Miller and Gasek (1960) fed the gradient container from a mixer-reservoir which initially contained the denser mixture; as this liquid was removed, the lighter mixture was gradually added. The density gradient container had constantly to be lowered to keep the inlet tube at the liquid surface of the gradient. The inlet tube could not be moved without changing the flow rate, since liquid was fed by gravity. This technique seems cumbersome since mixing rate,

flow rate and shifting of gradient container must either be carefully synchronised or continuously watched. It is difficult to control the shape of the gradient by this method.

Wolff (1975) used Pertoft's (1966) modification in which the lighter mixture is added first through a tube to the bottom of the container. By this method, gradient shape and mixing rate are easily controlled; the gradient may even be made in steps and allowed to diffuse to linearity. Flow rate is unimportant. The only disadvantage is that as the inlet tube is withdrawn, the gradient may be disturbed. If a continuous gradient is prepared, it may thus 'flatten out', by diffusion, more rapidly. If a step-wise gradient is prepared, the boundaries between the steps may be 'blurred'. The steps in gradients of this type, or in ultracentrifuge density gradients, should be as sharp as possible or the final gradient, after diffusion, will be sigmoid rather than linear (Dr. O.M. Griffiths, personal communication).

(ii) Drying of solvents

Commercial preparations of CCl_4 and petroleum ether contain traces of water and water-soluble compounds. These were removed before gradients were prepared by saturating the solvents with distilled water, separating the bulk water and finally by drying with finely divided calcium chloride.

(iii) Preparation of gradients

Because of the disadvantages of previous methods, as described above, two new methods of preparing gradients were devised. All gradients were prepared in stoppered measuring cylinders of 500 ml, 1 l or 2 l capacity. (The graduations on the measuring cylinder provide a useful scale for preparing calibration curves.)

It is worth noting that it proved impractical to feed gradients by a peristaltic pump. The silicone tubing used in the available pumps (LKB Perpex and Watson-Marlow HR Flow Inducers) was attacked by CCl_4 ; the tubing became opaque and slippery, swelled up and eventually ruptured. All gradients were hence prepared by gravity feed, for step-wise preparation, or by siphon, for continuous preparation of gradient.

Mixtures of CCl_4 and petroleum ether of particular densities were easily prepared; there was no observable volume loss on mixing and hence a plot of measured densities against proportions of either component is linear (see fig. 2.6).

Stepwise gradient preparation

CCl_4 , petroleum ether, or mixtures of the two, were placed in a dropping funnel and allowed to run slowly down the side of the gradient container. The densest mixtures were applied first and gradients were made up using five different mixtures (or 'steps') with constant differences in density between the steps. Various density ranges, depending on the samples studied, were used.

Gradients were allowed to diffuse for at 48 h at room temperature before use and remained stable for 2-4 weeks, if undisturbed.

Continuous gradient preparation

Continuous gradients were prepared using an LKB 'Ultragrad' gradient mixer and mixing valve (LKB Produkter AB, Sweden) (see fig. 2.7). Teflon (PTFE) tubing, resistant to attack by CCl_4 or Petroleum Ether, was used throughout. Flow of liquid was induced by syphoning, using a syringe to initiate the flow. The gradient mixer automatically

Fig. 2.6 DENSITY OF CCl_4 -PETROLEUM ETHER MIXTURES

NOTES

(i) Temperature = 20°C .

(ii) Mixtures were made using X ml of CCl_4 and (10-X) ml of Petroleum Ether.

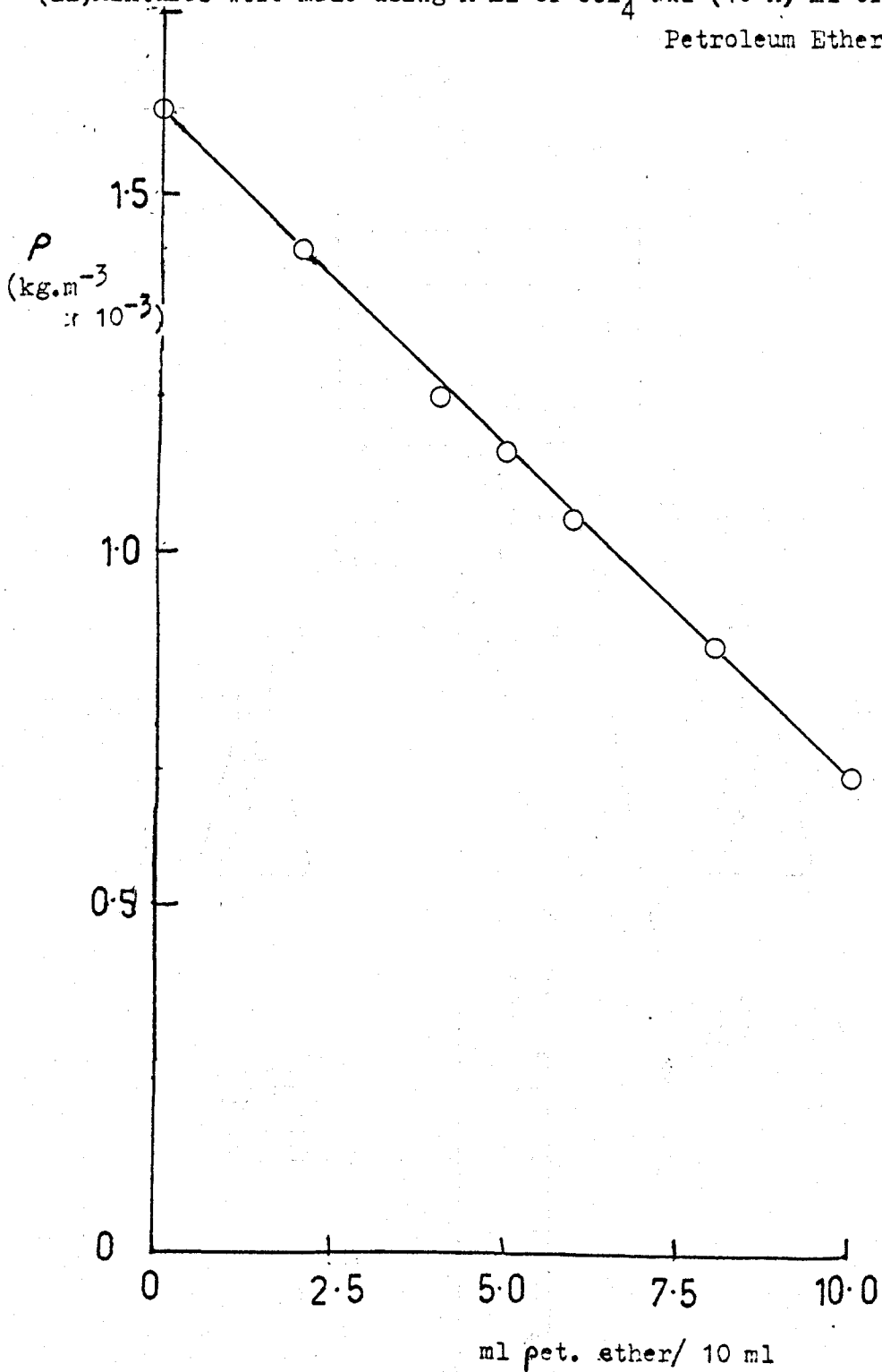
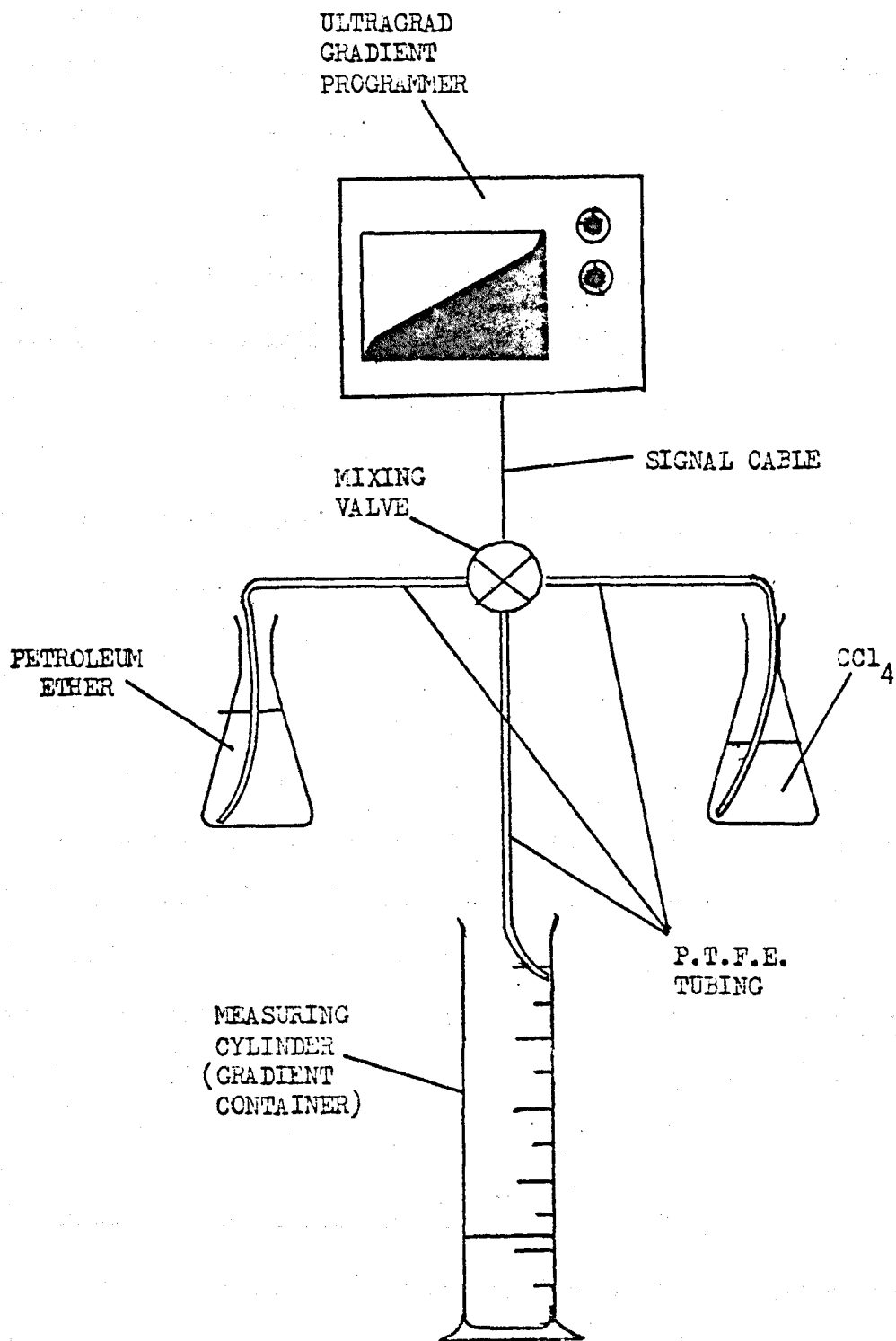


Fig. 2.7 CONTINUOUS PREPARATION OF ORGANIC SOLVENT DENSITY GRADIENTS



produced continuous gradients, so this process did not require constant attention. It was not necessary to pre-mix solutions of CCl_4 and petroleum ether, since any desired proportions of each component can be pre-programmed on the 'Ultragrad'. Finally, since the gradients are continuous, they can be used as soon as they are prepared. The preparation of one gradient takes less than 2h, even with the largest gradients used (2 litres).

(iv) Density standards

Four different solutions were used as density standards, sucrose, potassium iodide, potassium permanganate and copper sulphate. These were chosen for the density ranges available with solutions, and the last two because they would give coloured drops on the gradient; at low densities this colour was negligible, and solutions were stained with eosin which gives a deep red colour in salt solution and is insoluble in organic solvents. The concentration of eosin was insufficient to affect the measured densities of the solutions.

Solutions were made up in double distilled de-ionised water and stored in stoppered test-tubes; they were standardised by accurate weighing, on a Mettler H16 balance, of volumes, measured with individually calibrated pipettes or volumetric flasks, using double distilled deionised water as standard ($\rho_{\text{H}_2\text{O}}$ at 20°C and 101.325 kPa = $998.203 \text{ kg.m}^{-3}$). The shapes of the calibration curves were independent of the density standard used.

(v) Applications of this technique

This technique was primarily developed in order to study plaque ECF but its applications in oral biology are not limited to this. Sublingual saliva is produced in very small amounts (stimulated flow

rate = 0.01-0.1 ml/min; Stephen and Speirs, 1976) and is extremely viscous; it is impossible to pipette and has to be measured by weight, so the density of this secretion must be known in order to calculate volumes. The density gradient technique was therefore used to determine the density of sublingual saliva (Lindsay et al., 1978) and is being used to study other body secretions, in particular, the minor gland salivas which are also produced in small amounts (Darlington et al., manuscript in preparation).

2.3.7 Clearance of sucrose from saliva

Whole mouth saliva was collected by expectoration at various times during, and following, ingestion of various sweet foods and beverages. Saliva was collected in 15 ml centrifuge tubes in an ice bath and was centrifuged at 38,000 g for 20 min at 4°C to remove debris.

Saliva collected while beverages were still in the mouth was removed (by gravity flow) through a short piece of silicone tubing placed under the subject's tongue. Otherwise, saliva was collected into centrifuge tubes at 30 sec intervals. The detailed procedures for each of the various foods and beverages studied are listed below.

(i) 'Coca-Cola'

The subject drank 250 ml of 'Coca-Cola' ad lib over 0.5-1.0 min. Samples taken while the 'Coca-Cola' was in the mouth were shaken to remove dissolved CO₂.

(ii) Boiled sweet (Pascall's 'Fruit Drops')

The subject sucked a sweet (6.4 g) until it was completely dissolved (about 10 min). Saliva was collected in test-tubes during and for 10 min after this period. Samples were diluted as required and assayed for sucrose.

(iii) Toffee (Callard and Bowser; 'Cream-Line Toffee')

The subject chewed a toffee (8.4 g) until it was completely dissolved (about 2 min). Saliva was collected as in (ii). The samples contained large amounts of fat (from the sweet) and they were centrifuged at 38,000 \underline{g} at 4°C for 25 min to separate this fat.

(iv) Chocolate (Cadbury; 'Bournville Plain Chocolate')

Solid chocolate dissolved in the mouth more rapidly than other sweets (ii) and (iii) so the subject chewed 45 g of chocolate in six portions of about 7½ g each, over a period of 3.5 min. Saliva was collected as in (ii) and fat removed as in (iii).

(v) Sweetened Tea

The subject drank 150 ml of hot tea, containing milk and either 1 or 3 teaspoonfuls (6 or 18 g) of 'sugar' ('Analar' sucrose), ad lib over a period of 3 min. Saliva was collected as described above.

2.3.8 Clearance of sucrose from dental plaque

(i) Collection of plaque

The subject for these experiments was a healthy adult male volunteer who received no dental treatment during the course of the experiments. Plaque was grown for 24 h and experiments were performed 2 h after the last meal (breakfast). The subject rinsed his mouth for 30 seconds with 10 ml of a 20% w/v (584 mmol/l) solution of sucrose in distilled water. At various times after the rinse, plaque was removed, as quickly as possible, from all teeth using a nickel scraper and was placed in a humid plastic container in an ice bath. When all the plaque needed was collected, it was placed on a membrane filter [see next section (ii)] and weighed.

(ii) Separation of plaque extracellular fluid (ECF)

In previously published methods, plaque ECF was separated from plaque 'solids' by high speed centrifugation in a microhaematocrit centrifuge (Edgar & Tatevossian, 1971) or by lower speed centrifugation (5000 g) in wide bore centrifuge tubes (Tatevossian & Gould, 1976). With both methods, it is difficult to recover the separated fluid without contamination by bacterial cells. Various methods of isolating ECF were attempted in this study, but all were based on the following principle. Plaque was placed on a membrane filter (which would retain bacterial cells) and the extracellular fluid was drawn through the membrane by centrifugation.

Initially plaque was placed in 'Centriflo' molecular filter cones (Amicon Ltd., Woking, Surrey, England) and centrifuged, at 4°C, in the 'Centriflo' holders. However the volume of the 'Centriflo' apparatus was too large to allow convenient handling of the small plaque samples (30 mg or less). Moreover, the apparatus could only be used at low speeds (2,300 r.p.m., corresponding to 1,600 g). As a result, the rate of separation of the ECF was too low to be useful in the separation of plaque fluid (see fig. 2.8) which must be done very rapidly to avoid the risk of contamination by the contents of autolysed cells.

An apparatus was constructed (see fig. 2.9) to allow plaque fluid to be separated through 'Millipore' cellulose acetate membrane filter discs (diameter 13 mm; pore size 0.45 µm; Millipore (U.K.) Ltd., Park Royal, London, England). The fitted discs (with plaque samples) were held over a collecting centrifuge tube in a 'Swinnex' filter holder (Millipore). The whole assembly was centrifuged at 20,000 g, at 4°C, for 15 min. This speed and time removed about 30% of the weight of the plaque (see fig. 2.10) which corresponds roughly to the proportion of ECF in plaque reported by Edgar and Tatevossian (1971).

Fig.2.8 SEPARATION OF PLAQUE EXTRACELLULAR FLUID BY CENTRIFUGATION
THROUGH 'CENTRIFLO' MEMBRANE FILTERS

The symbols (◆) and error bars represent the means and standard deviations of values obtained with plaque from four different subjects.

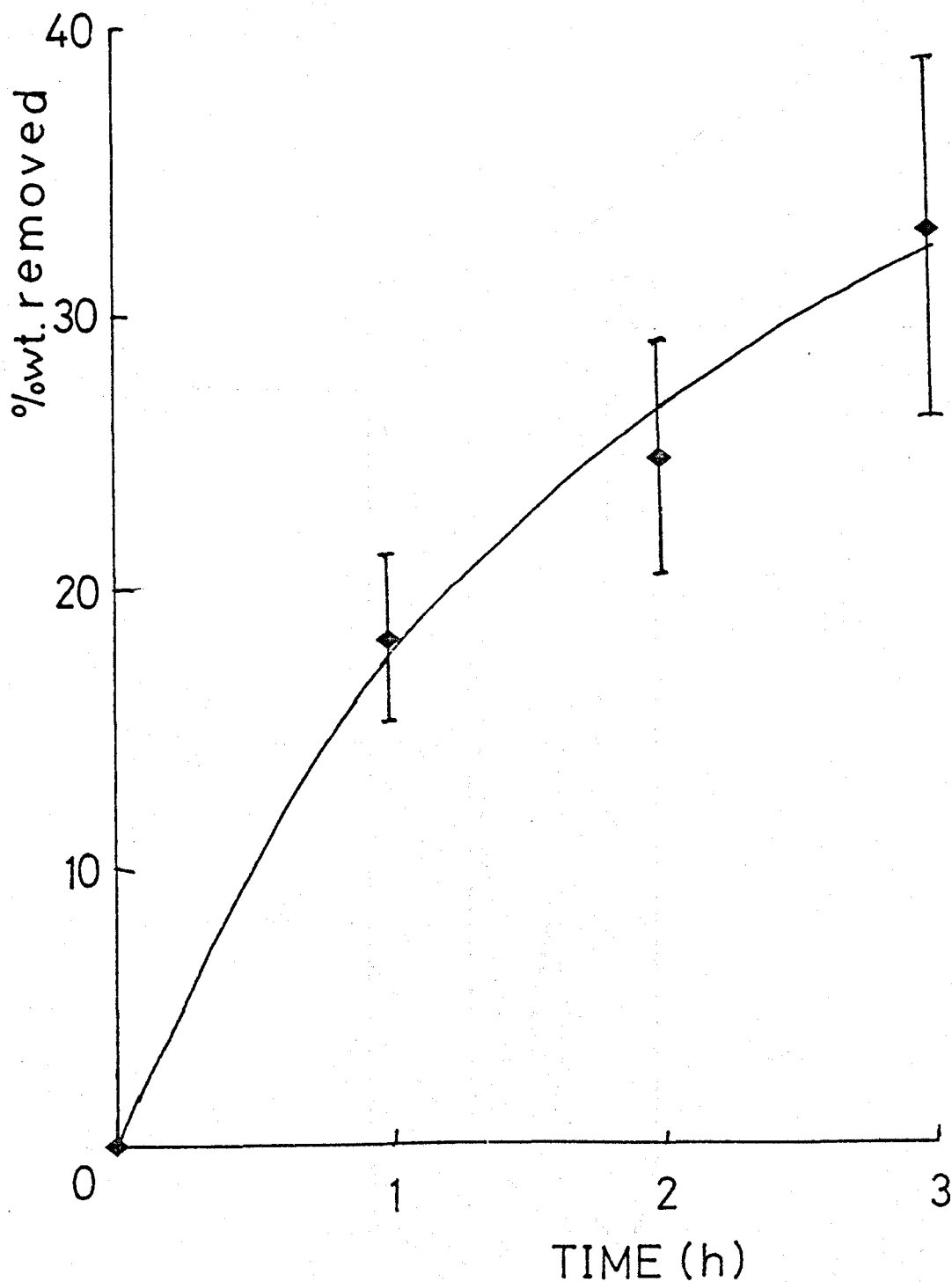


Fig.2.9 APPARATUS FOR SEPARATION OF PLAQUE EXTRACELLULAR FLUID
BY CENTRIFUGATION THROUGH 'MILLIPORE' MEMBRANE' FILTERS

The assembly was spun in a 45° degree fixed-angle rotor, so the direction of centrifugation was at 45° to the long axis of the apparatus, as shown by arrow (below).

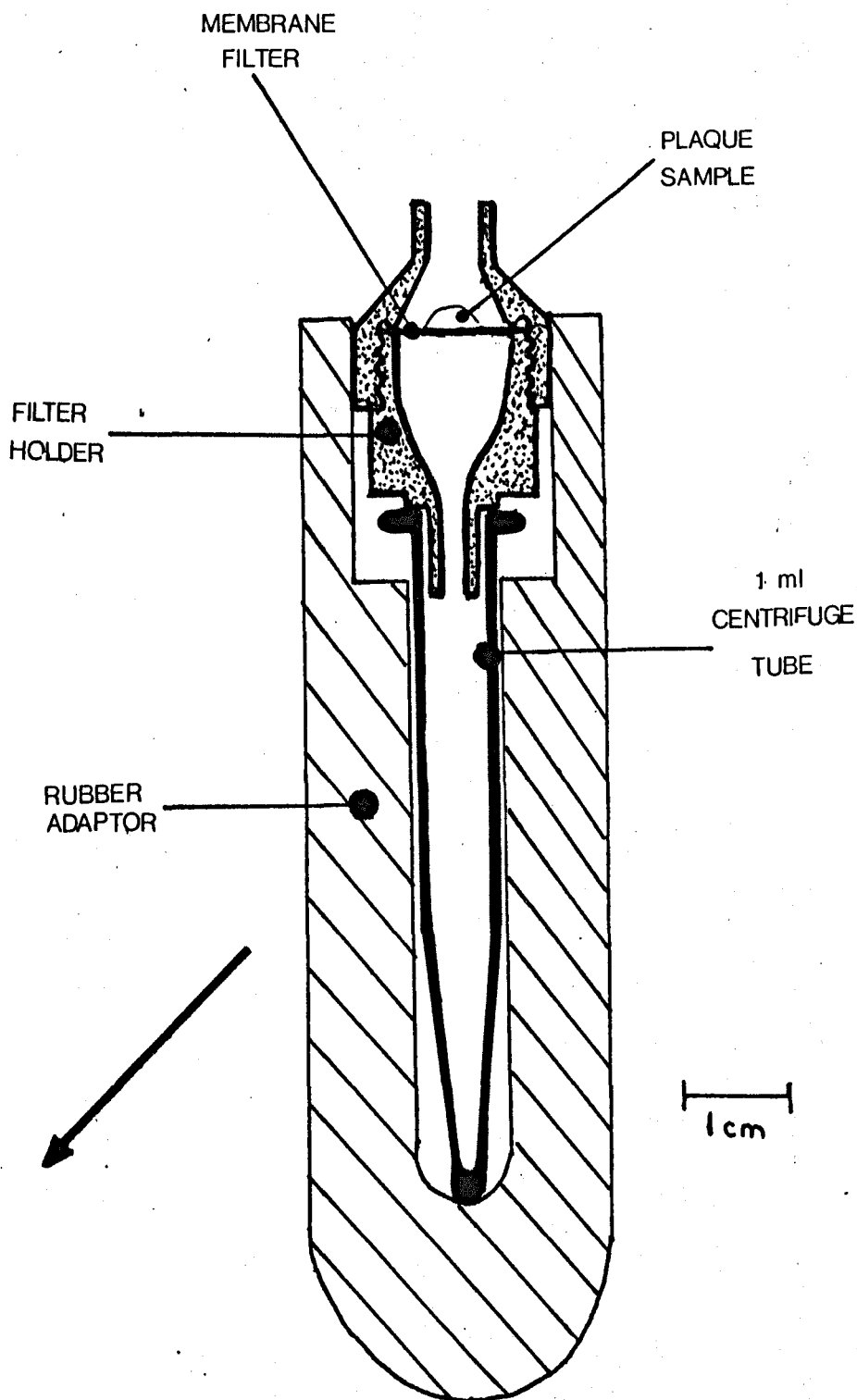
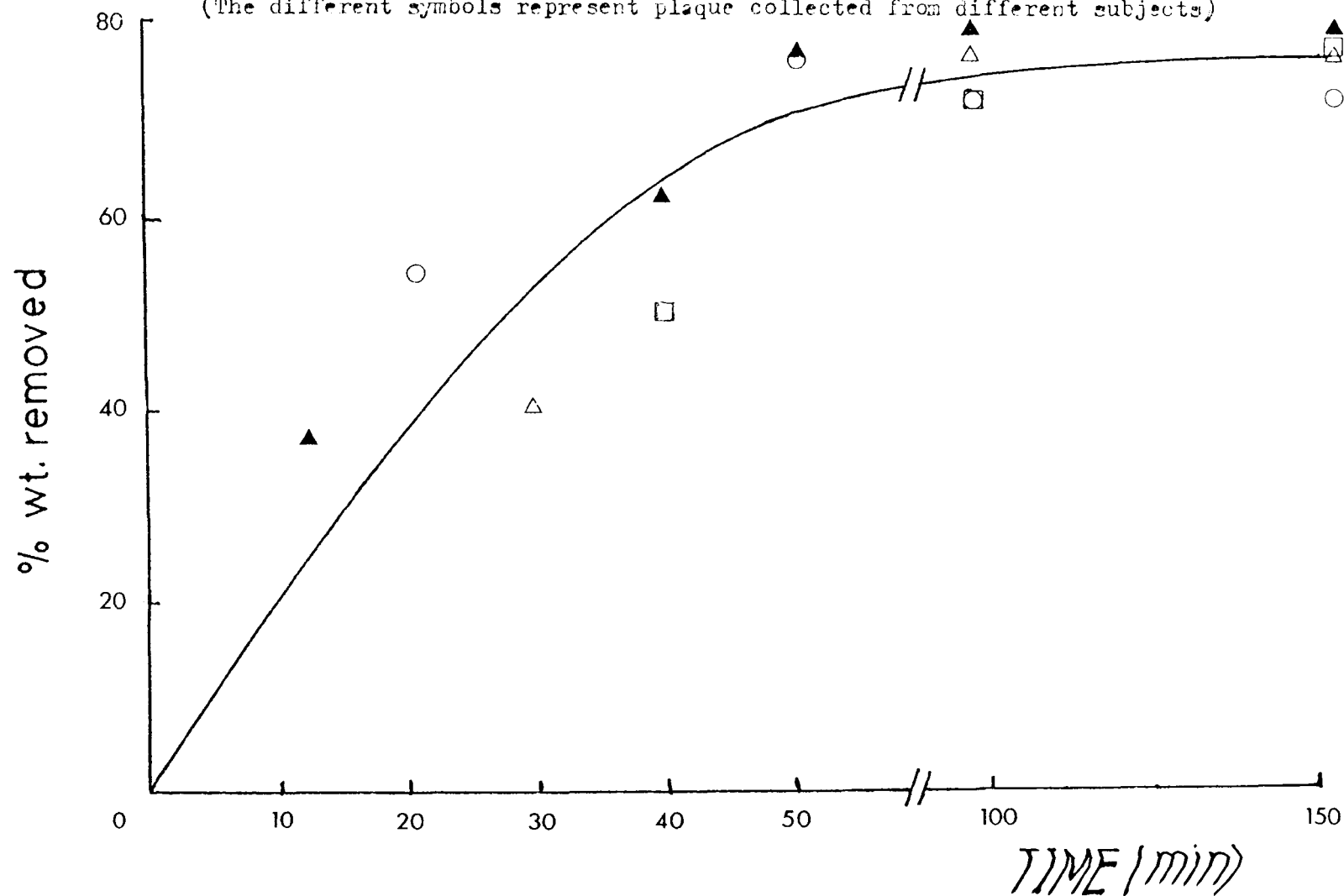


Fig.2.10 SEPARATION OF PLAQUE EXTRACELLULAR FLUID BY CENTRIFUGATION THROUGH 'MILLIPORE'
MEMBRANE FILTERS

(The different symbols represent plaque collected from different subjects)



In earlier experiments using the apparatus shown in fig. 2.9, the extracellular fluid presumed to have been collected in the centrifuge tube was diluted in 0.5-1.0 ml saline because the volumes of fluid (10 μ l or less) which could be collected from whole mouth plaque were too small to be conveniently pipetted. However, it was found that almost no fluid was observed in the collecting tube after centrifugation. Any extracellular fluid which passed through the membrane may have been retained, dried, on the bottom part of the filter holder. To overcome this, the plaque samples on the membrane were overlayed with varying amounts of 0.9% w/v NaCl (154 mmol/l) (0.2-0.5 ml). This was to ensure that any extracellular fluid would be thoroughly rinsed from the plaque into the centrifuge tube.

The diluted plaque ECF was then immediately assayed for sucrose and glucose concentrations, as described previously (Section 2.3.3). From these determinations, the amounts of sucrose and glucose in the original plaque sample could be calculated (expressed as nmoles per mg wet weight of plaque). The density of the plaque was determined ($1.04 \times 10^3 \text{ kg/m}^3$; see 3.8.2). Using this and the value of 31.2% (v/v) reported by Edgar and Tatevossian (1971) for the proportion of ECF in plaque, tentative values for the concentration of sucrose and glucose, in plaque ECF, were calculated.

3. RESULTS

3.1 Batch culture of *S. sanguis* 804

3.1.1 Production of extracellular glucosyltransferases

The project described in this thesis was based on the work of Beeley and Black (1977). They showed that, in batch cultures, in the absence of sucrose, *S. sanguis* 804 produces extracellular glucosyltransferases in three phases. It was thus initially necessary to determine whether or not this triphasic pattern could be confirmed.

Cultures of *S. sanguis* 804 were grown at constant pH (7.0 ± 0.1) as described by Beeley and Black (1977; see 2.3.1). Fig. 3.1 shows the results from one such culture. The three phases of enzyme activity, corresponding to those described by Beeley and Black, are indicated. Phase I occurs at the onset of logarithmic growth, and phase II at the start of stationary phase, when the growth medium was depleted of glucose; both were clearly defined. Phase III, which occurs 30-40 hours into stationary phase, was not examined.

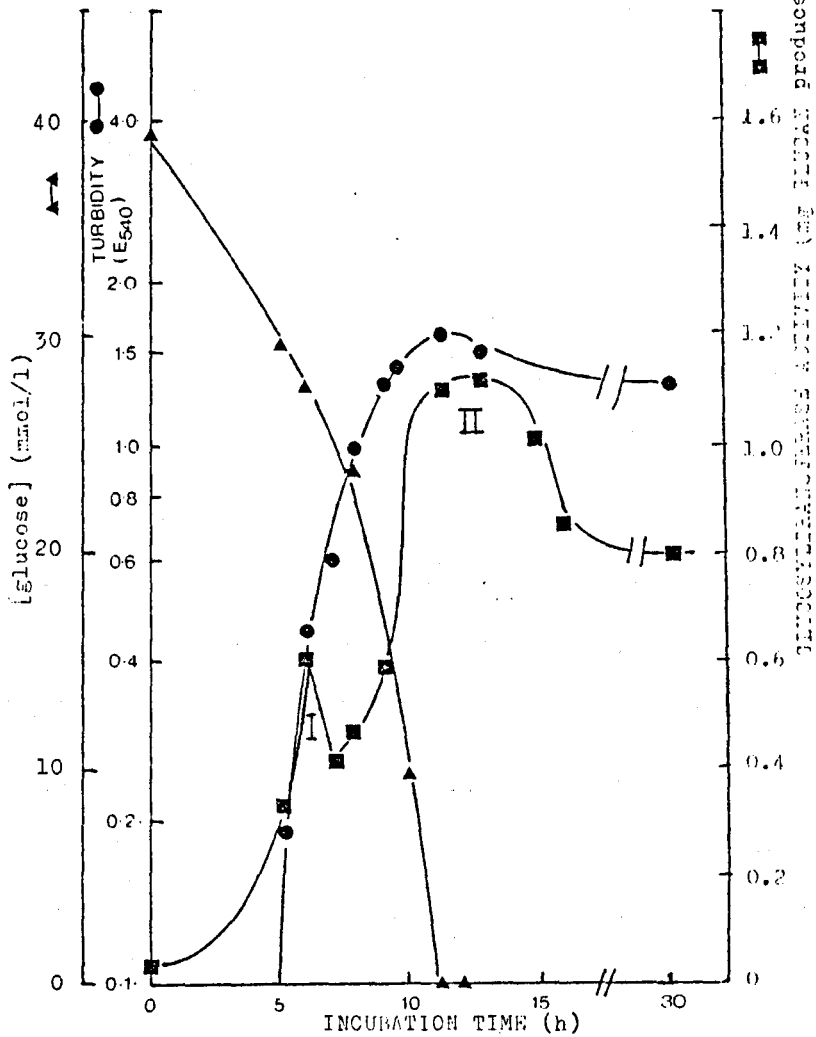
Rate and extent of cell growth, and amounts and specific activities of glucosyltransferases produced were all similar to those described by Black (1975) and Beeley and Black (1977). The concentration of protein in the culture medium, about 2.5 g/l as measured by the Lowry method (see Section 2.3.3), did not vary during the course of the culture.

In a similar experiment using the same growth medium, Carlsson and Elander (1973) showed that *S. sanguis* 804 produced optimum amounts of glucosyltransferase activity when the medium contained 1% w/v glucose (55.5 mmol/l). In batch culture, using similar conditions (pH 7.0, 35°C,

Figure 3.1

EXTRACELLULAR GLUCOSYLTRANSFERASE PRODUCTION

BY *S. sanguis* 804 IN BATCH CULTURE



NOTES

- (i) Culture volume was 250 ml.
- (ii) 5 ml samples were withdrawn at various time intervals.

1% w/v glucose) to those described in this thesis, the values of the various growth parameters were similar to those reported here. Carlsson and Elander did not find a triphasic pattern of glucosyltransferase production but they did not study the early (less than 6 h incubation) or late (greater than 12 h) stages of growth. Thus, the first and third phases of glucosyltransferase production would have been overlooked.

3.1.2 Acid production

Phase II of glucosyltransferase production (as described in 3.1.1) was studied in this project (see also Section 1.4 - 'Aims of this Study'). Phase II reaches a peak when the medium is depleted of glucose and cell growth ceases. Both of these parameters can be monitored fairly easily. However, measurements of cell growth as increases in turbidity become less reliable at high cell numbers because the bacteria begin to aggregate (Black, 1975) and because samples must be diluted before turbidity is measured. Glucose concentrations can be estimated rapidly and semi-quantitatively by 'Clinistix' reagent strips (Ames Co., Slough, England) but these are too sensitive to permit one to monitor the earlier stages of cultures. Glucose concentrations can be accurately and reliably determined by the glucose oxidase-Perid method (see 2.3.3). However, this is time-consuming and, by the time results are obtained, the peak of glucosyltransferase activity in phase II may have been passed. It was thus necessary to develop a rapid and reliable method for identifying the peak of phase II, in order to harvest the maximum possible amount of glucosyltransferase activity.

S. sanguis 804 produces large amounts of acid by fermenting glucose, under the culture conditions used. Addition of NaOH, necessary

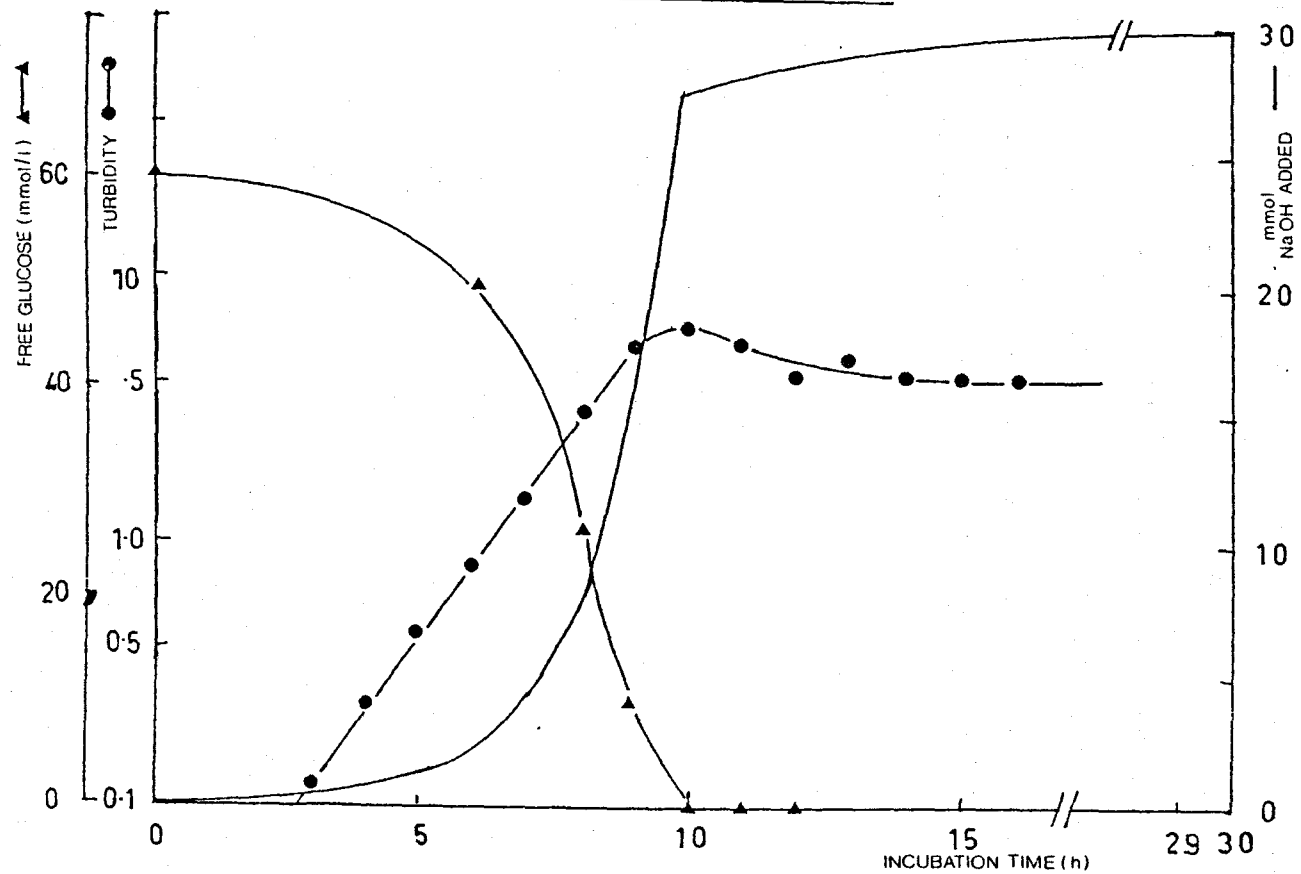
to maintain the pH at 7.1 ± 0.1 , was monitored by a chart recorder, coupled to the pH-stat apparatus, as described in section 2.3.1. Fig. 3.2 shows that the rate of acid production closely followed the rates of cell growth and depletion of glucose from the medium. The rate of acid production and cell growth decreased sharply when the medium was depleted of glucose; this point corresponds to the peak of phase II (see Section 3.1.1 and Beeley & Black, 1977). Beyond this point, the rate of further acid production, probably due to release of acid from autolysed cells, was extremely low.

In this culture, about two equivalents of titratable acid were produced for every mole of glucose in the culture medium (see Fig 3.2). The acids produced have not been identified. If, as in dental plaque (see 1.3.1), most of the acid produced is lactate, then this 2:1 ratio seems reasonable. However, it is possible that other organic acids and some carbon dioxide were also produced. Using the same technique, other workers have also found that approximately two moles of acid are produced for every mole of glucose, in these cultures (Lundie, 1978; H. Parton, personal communication).

Thus, monitoring of acid production was useful for determining the peak of phase II of glucosyltransferase production and was used for this purpose in most cultures.

This technique has also subsequently been used to measure acid production by S. sanguis 804 from xylitol (Beeley et al., 1978) and other sugars (Lundie, 1978). This is an extremely sensitive method of estimating 'acidogenicity' of various sugars. (Acidogenicity is one of the principal characteristics of cariogenic foodstuffs; see 1.3.1.) Other workers have measured the fall in pH in batch culture (e.g. Hayes

Figure 3.2 ACID PRODUCTION BY *S.sanguis* 804 IN BATCH CULTURE



NOTES

- (i) Culture volume was 250 ml. Total amount of glucose = 15 mmoles.
- (ii) 5 ml samples were withdrawn at various time intervals.

& Roberts, 1978) or have titrated the acids produced in cultures grown without pH control. These techniques have the disadvantage that bacterial growth and metabolism are self-limited by the low pH produced. The value of the final pH in such cultures is dependent upon the pK of the acids produced and upon any buffering compounds in the incubation medium. It is thus difficult to relate the change in pH to the absolute amounts of acid produced, and, hence, to the amount of sugar metabolised. When acid production is monitored directly, in a culture at constant pH, the amount of acid produced will be directly proportional to the amount of sugar metabolised.

3.2 Purification of Glucosyltransferases

(All purification procedures were as described in Section 1.3.4)

3.2.1 Ammonium sulphate fractionation

In this study, the volumes of culture supernatant used (up to 4 l) were often too large for easy handling. Moreover, because the bacteria were grown in a diffusate medium, many oligopeptides were present in culture fluids and these might complicate purification of the glucosyltransferases. Ammonium sulphate precipitation was studied to determine whether or not the glucosyltransferases in culture fluid could be concentrated into a small volume and whether or not the oligopeptide material could be removed.

In the experiments shown in Tables 3.1 and 3.2, almost all of the enzyme activity recovered (98% of total recovery) was precipitated by 70% saturated $(\text{NH}_4)_2\text{SO}_4$. Most of the 'protein' (98-99% of the recovery) remained in the supernatant. Much of the unprecipitated material may have been oligopeptide. As the two experiments show, the degree of purification obtained varied considerably (40-100-fold purification). This is probably because such a small proportion of the total protein was actually precipitated. The apparent specific activity of the glucosyltransferases will thus be sensitive to slight changes in the amounts of protein precipitated. These preliminary studies showed that the glucosyltransferases could not only be concentrated but were also extensively purified by this single procedure.

Many proteins, other than the glucosyltransferases, may also be precipitated by 70% saturated ammonium sulphate. It was, however, not feasible to separate these further by selecting 'cuts' at different

Table 3.1 AMMONIUM SULPHATE FRACTIONATION OF GLUCOSYLTRANSFERASES 0 - 70% SATURATION

PREPARATION	VOLUME (ml)	TOTAL PROTEIN (mg) [% age RECOVERY]	TOTAL GLUCOSYLTRANSFERASE ACTIVITY (mg CHO↓/24h) [% age RECOVERY]	SPECIFIC ACTIVITY (mg CHO↓/24/mg protein) [PURIFICATION]
(1) Culture Supernatant	100	218 [100]	66.4 [100]	0.341 [1]
(2) Precipitate (0-70% saturated ammonium sulphate)	3.0	4.49 [2.6]	59.4 [89.5]	13.2 [38.8]
(3) Dialysed ammonium sulphate supernatant	205	41.8 [19.2]	1.3 [1.96]	0.031 [0.091]
(4) Diffusate from ammonium sulphate supernatant	1470	147 [67.5]	0 [0]	0 [-]
TOTAL YIELD (2)+(3)+(4)	-	193 [89]	60.7 [91]	- [-]

NOTES

- (i) (3) and (4) together are the material not precipitated by ammonium sulphate. (3) is the dialysed material and (4) is the diffusate from this by dialysis in visking tubing.
- (ii) Protein was estimated by the method of Lowry as described in section 2.3.3.

Table 3.2

AMMONIUM SULPHATE FRACTIONATION OF GLUCOSYLTRANSFERASES - 0 - 70% SATURATION

PREPARATION	VOLUME (ml)	TOTAL PROTEIN (mg) [% age RECOVERY]	TOTAL GLUCOSYLTRANSFERASE ACTIVITY (mg CHO↓/24 h) [% age RECOVERY]	SPECIAL ACTIVITY (mg CHO↓/24/mg protein) [PURIFICATION]
(1) Culture supernatant	180	709 [100]	594 [100]	0.838 [1]
(2) Precipitate (0-70% saturated ammonium sulphate)	5.0	5.25 [0.71]	440 [74]	83.8 [100]
(3) Dialysed ammonium sulphate supernatant	343	202 [28]	8.9 [1.5]	0.037 [0.044]
(4) Diffusate from ammonium sulphate supernatant	2480	273 [38]	0 [0]	0 [-]
TOTAL YIELD (2)+(3)+(4)	-	480 [68]	449 [76]	- [-]

NOTES: As Table 3.1.

concentrations of ammonium sulphate. Glucosyltransferase activity was precipitated over the whole range of ammonium sulphate concentrations (see table 3.3). This broad precipitation range is consistent with suggestions that 'glucosyltransferase' is a multi-enzyme system.

However, although the glucosyltransferases can be greatly concentrated (30-fold or more) by precipitation with ammonium sulphate, for very large culture volumes, this procedure would be still unwieldy (and expensive in purified ammonium sulphate). Therefore, culture supernatants were concentrated and partially desalted in a Bio-Fiber 80 hollow fibre device (mol.wt. cut-off 30,000) before ammonium sulphate precipitation. The results of one preparation using this technique are presented in Table 3.4. A considerable amount of low molecular weight protein and oligopeptides (together, 70% of the total protein present) were removed by this procedure. Essentially no glucosyltransferase activity passed through the pores of the hollow fibres, so the molecular weights of the enzymes (or any multimeric forms) are probably greater than 30,000. Glucosyltransferase was purified 182-fold after subsequent ammonium sulphate precipitation. This was even more than the best results without the ultrafiltration step. The reason for this is not clear. In the example shown, culture supernatant was concentrated 2-fold; in other experiments, supernatant was easily concentrated 4 to 5-fold.

In all the experiments described above, fairly small volumes of culture fluid were used in order to investigate the usefulness of ammonium precipitation. However, glucosyltransferase was successfully purified, with similar recoveries and degrees of purification, using much larger volumes (up to 4 l).

Table 3.3 AMMONIUM SULPHATE FRACTIONATION OF GLUCOSYLTRANSFERASES -

SUMMARY CHART

FRACTION	% of Total protein recovered	% of Total glucosyl-transferase recovered
PRECIPITATE (0-30% saturated ammonium sulphate)	0.6	42
PRECIPITATE (0-63% saturated ammonium sulphate)	0.9	97
PRECIPITATE (0-70% saturated ammonium sulphate)	1.7	98
UNPRECIPITATED MATERIAL (0-70% saturated ammonium sulphate)	98	1.7

NOTES

(i) This table is a summary of the results of different experiments.

Table 3.4 AMMONIUM SULPHATE PRECIPITATION OF GLUCOSYLTRANSFERASES - FOLLOWING HOLLOW-FIBRE ULTRAFILTRATION

PREPARATION	VOLUME (ml)	TOTAL PROTEIN (mg)	[% age RECOVERY]	TOTAL GLUCOSYLTRANSFERASE ACTIVITY (mg CHO↓/24 h)	[% age RECOVERY]	SPECIFIC ACTIVITY (mg CHO↓/24h/mgprotein)	[PURIFICATION]
(1) Culture supernatant	190	531	[100]	152	[100]	0.287	[1]
(2) Ultrafiltered culture supernatant	95	162	[30.5]	134	[88]	0.828	[2.9]
(3) Ultrafiltrate from culture supernatant	160	365	[68.8]	2.59	[1.7]	0.0071	[0.025]
(4) Precipitate from ultrafiltered culture supernatant (0-70% saturated ammonium sulphate)	5.3	3.67	[0.69]	192	[>100]	52.3	[182]
(5) Material unprecipitated from culture supernatant (dialysed)	230	40.5	[7.6]	2.09	[1.4]	0.0516	[0.180]
(6) Diffusate from unprecipitated material	1150	60.0	[11.3]	18.6	[12.2]	0.311	[1.0]
TOTAL YIELD (3)+(4)+(5)+(6)	-	469	[88]	215	[>100]	-	[-]

3.2.2 Hydroxylapatite chromatography

(i) Introduction

The extracellular glucosyltransferases of S. sanguis and S. mutans bind strongly to hydroxylapatite (HA) and HA chromatography has been used previously to purify them. Those of S. sanguis 804 have been purified using step-wise gradient elution (0.2 mol/l, pH 6.0, to 0.5 mol/l, pH 6.8 phosphate buffer) (Carlsson et al., 1969; Black, 1975). Those of S. mutans were eluted over the same range (0.2-0.5 mol/l, pH 6.0) (Guggenheim & Newbrun, 1969) or at even lower concentrations of phosphate (0.06 mol/l, pH 6.0 phosphate buffer) (Chludzinski et al., 1974).

Most proteins are eluted from hydroxylapatite by about 0.4 mol/l phosphate or less. This, and the strong binding of glucosyltransferases to HA described in the preceding paragraph, suggested that HA chromatography would be useful in further purifying the ammonium sulphate preparations described in section 3.2.1. Bernardi et al. (1972) showed that binding of proteins to HA could be weakened by even slight decreases in pH. Thus, in order to maximise binding of proteins and perhaps improve resolution in elution profiles, all chromatography buffers were at pH 6.8. Potassium phosphate buffers were used because at 4°C, sodium phosphate buffers are not sufficiently soluble at the higher concentrations (up to 1.0 mol/l) used.

(ii) Step-wise gradient elution

In a first attempt to purify the glucosyltransferases, protein was eluted from a column of hydroxylapatite by a stepwise gradient of potassium phosphate buffer (pH 6.8) from 0.001 to 1.0 mol/l (see Fig 3.3).

Fig 3.3 HYDROXYLAPATITE CHROMATOGRAPHY OF $(\text{NH}_4)_2\text{SO}_4$ PURIFIED

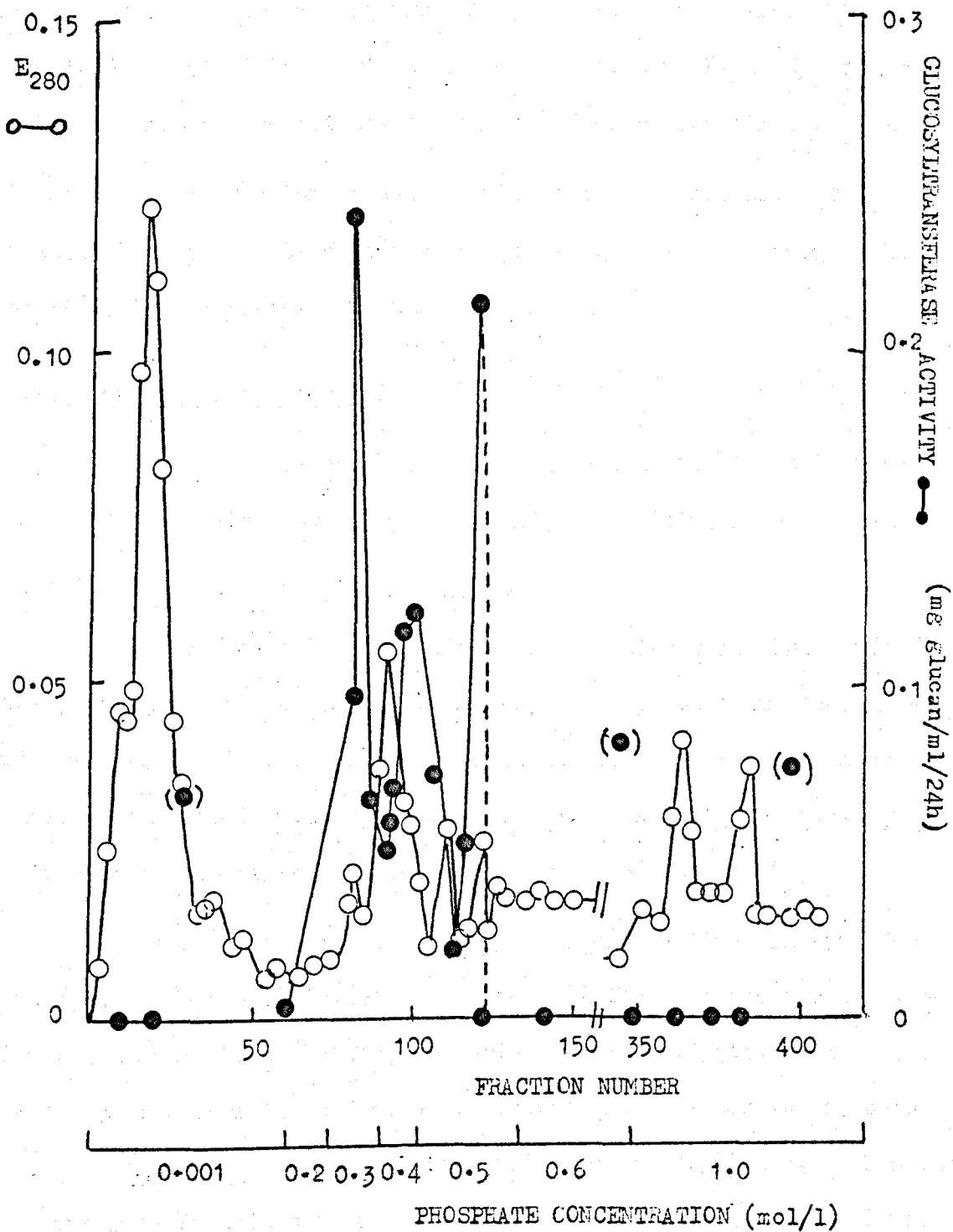
GLUCOSYLTRANSFERASES - STEPWISE GRADIENT ELUTION

NOTES

- (i) Sample volume= 1.0 ml. Total sample protein= 1.5 mg. Total sample glucosyltransferase activity= 19.8 mg precipitable glucan/ 24 h.
- (ii) Flow rate= 36 ml/h. Fraction volume= 3.6 ml.
- (iii) Column bed volume= 39 ml (13 mm height x 63 mm diam).

Fig. 3.3 HYDROXYLAPATITE CHROMATOGRAPHY OF $(\text{NH}_4)_2\text{SO}_4$ PURIFIED

GLUCOSYLTRANSFERASES - STEPWISE GRADIENT ELUTION



Fractions eluted at high phosphate concentrations were diluted 10-fold before assay for enzyme activity, to minimise any possible inhibition by high ionic strength. The step-wise gradient eluted several, poorly resolved peaks of enzyme and protein. The protein (UV-absorbing material) was still being eluted, even up to 1.0 mol/l phosphate, and peaks were not always coincident with peaks of glucosyltransferase activity.

This particular procedure, therefore, is probably not practicable for isolation of any individual glucosyltransferases and was not investigated further.

(iii) Continuous gradient elution

Once it is set up, a continuous gradient of eluant is more easily run than a step-wise gradient and, ultimately, resolves peaks more clearly.

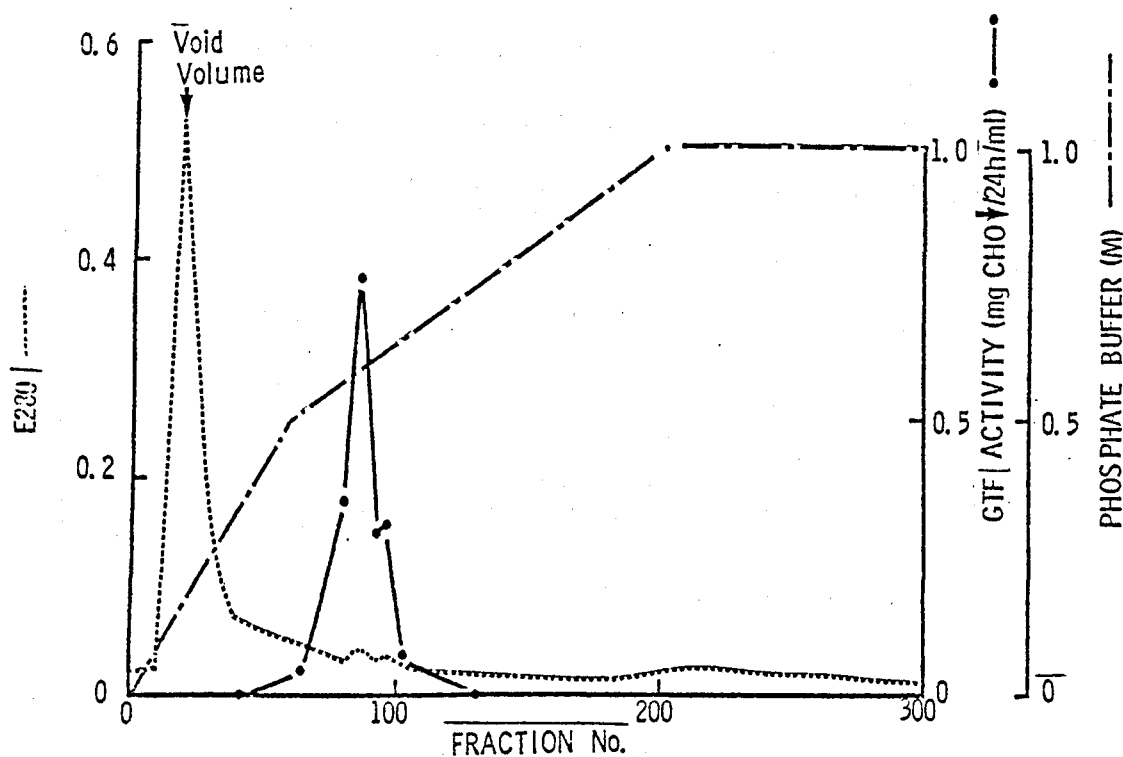
In the experiments reported here, samples were eluted with continuous gradients of 0.001-1.0 mol/l, pH 6.8, potassium phosphate buffer. Gradients were produced using an 'Ultragrad' electronic gradient mixer (LKB Produkter, AB, Sweden). With this device, any desired shape of gradient could be programmed.

In earlier experiments, the number of fractions assayed for glucosyltransferase activity was severely limited because the assay method of Cybuska and Pakula (1963a), measuring glucan production, was used. The assay procedure takes 3 days to complete and the assays and zero time controls, for each sample, are performed in triplicate. This limits the number of fractions which can be assayed at any one time. It is also difficult to compare enzyme activities of any fractions which are selected for further assays, with activities of fractions assayed earlier,

since some activity will be lost during storage. It was thus not possible to distinguish clearly different peaks of enzyme activity eluted from hydroxylapatite. However, the results given in fig 3.4 show that the glucosyltransferases constitute only a small proportion of the total protein precipitated by ammonium sulphate. Most of the protein had no glucosyltransferase activity and was eluted with void volume of the column. All of the glucosyltransferase activity recovered had bound to the column and was eluted between 0.5 and 0.7 mol/l phosphate. Glucosyltransferases were not resolved as peaks of protein or enzyme activity mainly because only a very small amount of protein was applied to the column. It is possible that the poor resolution was due also to the limitations on numbers of fractions assayed for enzyme activity and to the very low E_{280} (near background) of eluted material. However, in spite of this, it was clear that continuous gradient elution would satisfactorily separate the glucosyltransferases from the bulk of the protein present.

Fig. 3.5 shows the results of hydroxylapatite chromatography with a much larger amount of protein (obtained from a 4 l culture of S. sanguis 804) applied to the column. In this experiment, the proportion of unadsorbed protein was smaller with respect to the amount of adsorbed protein than in the experiment shown in fig. 3.4. This is because, in this case, the redissolved ammonium sulphate precipitate was dialysed in a Bio-Fiber 80 device, against the running buffer, to remove any remaining ammonium sulphate. Some low molecular weight (less than 30,000) proteins and residual oligopeptides may also have been removed by this process although their concentrations in the diffusate would have been too low to measure accurately. Moreover,

Fig. 3.4 Hydroxylapatite chromatography of $(\text{NH}_4)_2\text{SO}_4$ purified glucosyltransferases of *S. sanguis* 804 — Continuous gradient elution.



NOTES

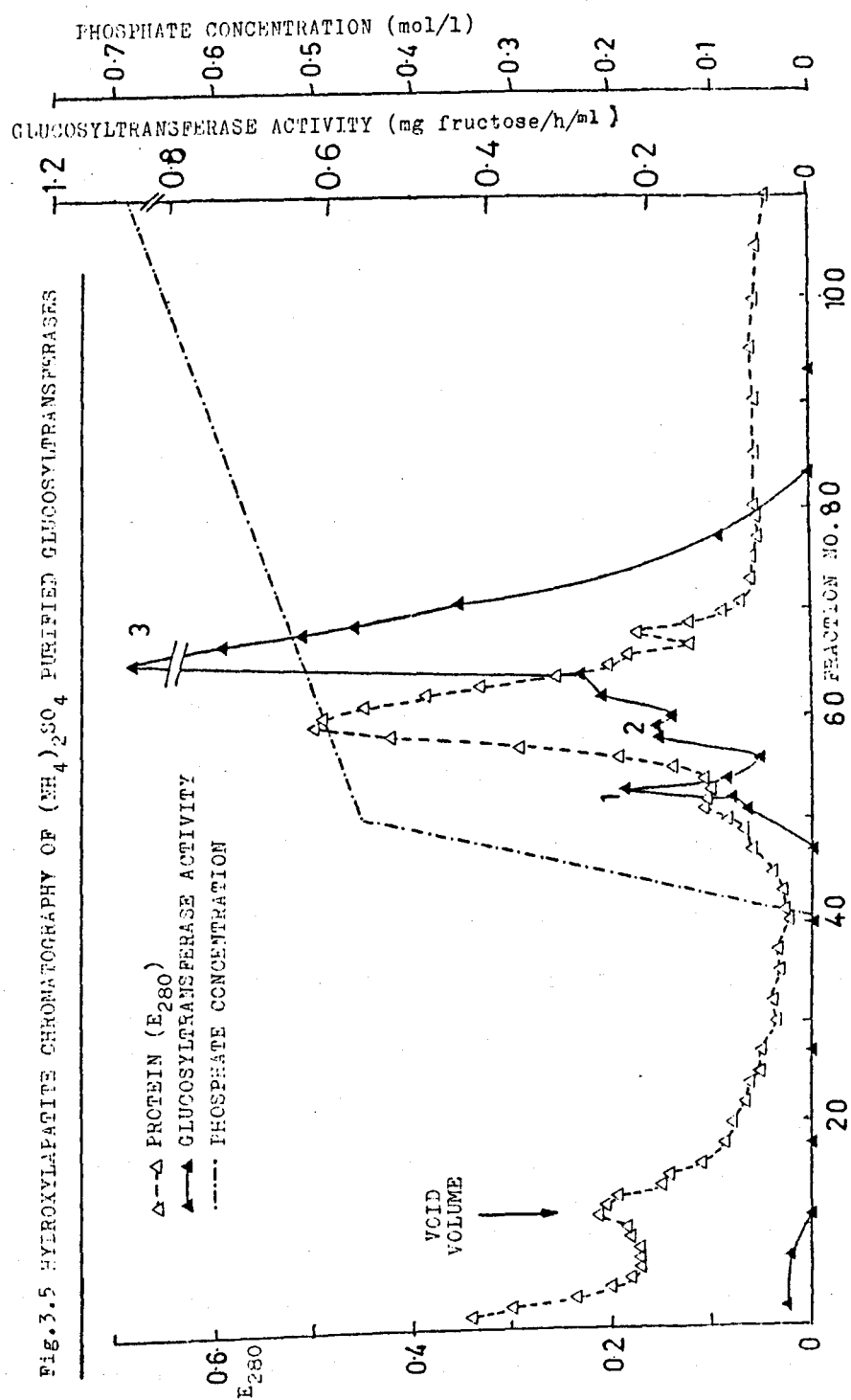
- (i) Sample volume= 3.0 ml. Total sample protein= 4.8 mg. Total sample glucosyltransferase activity= 52 mg precipitable glucan/24 h.
- (ii) Flow rate= 36 ml/h. Fraction volume= 3.6 ml.
- (iii) Column bed volume= 62 ml (20 mm height x 63 mm diam).

Fig. 3.5 HYDROXYLAPATITE CHROMATOGRAPHY OF $(\text{NH}_4)_2\text{SO}_4$ PURIFIED

GLUCOSYLTRANSFERASES — CONTINUOUS GRADIENT ELUTION

NOTES

- (i) Sample volume= 40 ml. Total sample protein= 7.04 mg. Total sample glucosyltransferase activity= 235 mg fructose/ h. (N.B. In the experiments shown in figs 3.3 & 3.4, glucosyltransferase activity was measured as the rate of synthesis of precipitable glucan; in this experiment, it was measured as the rate of release of fructose)
- (ii) Flow rate= 34.3 ml/h. Fraction volume= 8.02 ml.
- (iii) Column bed volume=114.5 ml. (18 mm height x 90 mm diam).
- (iv) Recovery of enzyme activity= 30% (70 mg fru/h).



because of the lower salt concentration after dialysis, some proteins, not previously adsorbed, may have bound to the column. Glucosyltransferase activity was assayed by the method of Carlsson (1969), measuring release of fructose from sucrose. In practical terms, using this technique allows one to assay a greater number of fractions. This technique is probably also more sensitive in detecting minor peaks of enzyme activity. Germaine et al. (1974) showed that glucan synthesis by S. mutans glucosyltransferases became very dependent on added dextran, after the enzymes were purified by hydroxylapatite chromatography. They concluded that acceptors and/or primers for glucan synthesis were removed by hydroxylapatite chromatography. Thus, enzymes eluted from hydroxylapatite would initially synthesise oligosaccharides, using sucrose as the first glucosyl acceptor (see Section 1.3.4). Minor peaks might not be detected by assays for precipitable glucan production, but would be detected by assays for release of fructose.

In this experiment, glucosyltransferase activity and adsorbed protein were eluted over the range 0.45 to 0.55 mol/l phosphate. Glucosyltransferases were eluted as three distinct peaks, two minor at 0.46 and 0.48 mol/l phosphate and one major at 0.51 mol/l phosphate with 'shoulders' on either side of the major peak. Protein was eluted as three peaks, 2 minor at about 0.45 and 0.52 mol/l phosphate and 1 broad major peak at 0.48 mol/l phosphate with a 'shoulder' at about 0.50 to 0.51 mol/l phosphate. The major peak of protein was co-eluted with the second peak (peak 2 in fig. 3.5) of glucosyltransferase activity. The other two peaks were eluted very close to (but not perfectly coincident with) peaks 1 and 3 of glucosyltransferase activity. Fractions of peak glucosyltransferase activity (peak 1, fractions 49-53; peak 2, 57-63; peak 3, 65-73) were pooled, dialysed against water, and freeze-

dried, as soon as possible to avoid loss of enzyme activity. As a result, the concentrations of protein in individual fractions were not determined and no exact measurement of specific activity was possible. However, a rough idea of specific activity may be calculated as enzyme activity divided by E_{280} ;

Glucosyltransferase	'Specific Activity'
Peak	(mg fructose/h/ml/ E_{280})
1	2.26
2	0.36
3	5.69

The area under the curve of glucosyltransferase activity was measured by means of a planimeter. According to this, the recovery of enzyme activity was about 30% of that applied to the column.

(iv) Conclusions

The extracellular glucosyltransferases precipitated by ammonium sulphate were further extensively purified by hydroxylapatite chromatography. No exact value for the degree of purification was obtained but the results shown in fig. 3.4 suggest that the enzymes were purified at least another 50-fold. The apparent recovery of enzyme (about 30%) as shown in fig. 3.5 is perhaps artificially low since one might expect hydroxylapatite chromatography to remove any glucosyl acceptors present in the material precipitated by ammonium sulphate.

Three different enzyme peaks of differing specific activities were detected. There may be other minor peaks present, but these could not be resolved because the peaks of enzyme activity overlapped considerably. The peaks could be separated by using a shallower elution gradient or by automatically programming the gradient to level off as

soon as UV absorbing material is eluting. In the latter method, the LKB 'Level Sensor' is connected between a flow-through monitor for UV-extinction, such as a 'Uvicord' (LKB), and the LKB 'Ultragrad' electronic gradient programmer. Whenever the UV absorption of eluted material reached a pre-set level, the Level Sensor would switch the gradient programmer to maintain a constant concentration of eluant. The concentration would not start to increase again until the peak of UV absorbing material was completely eluted. Further separation of the peaks would also permit better recovery of individual enzymes. In the experiments described here, for example, only a narrow range of fractions were pooled for freeze-drying to minimise any cross-contamination due to overlapping of the peaks.

It has been shown here that hydroxylapatite chromatography can be used to purify individual glucosyltransferases but this was not investigated further. A major aim of the project was to determine the properties of the glucosyltransferase system as a whole and the ammonium sulphate purified material was adequate for this (as well as being more convenient in practical terms and probably more relevant with respect to the biochemistry of plaque).

3.2.3 Experiments on affinity chromatography on Sephadex-G10

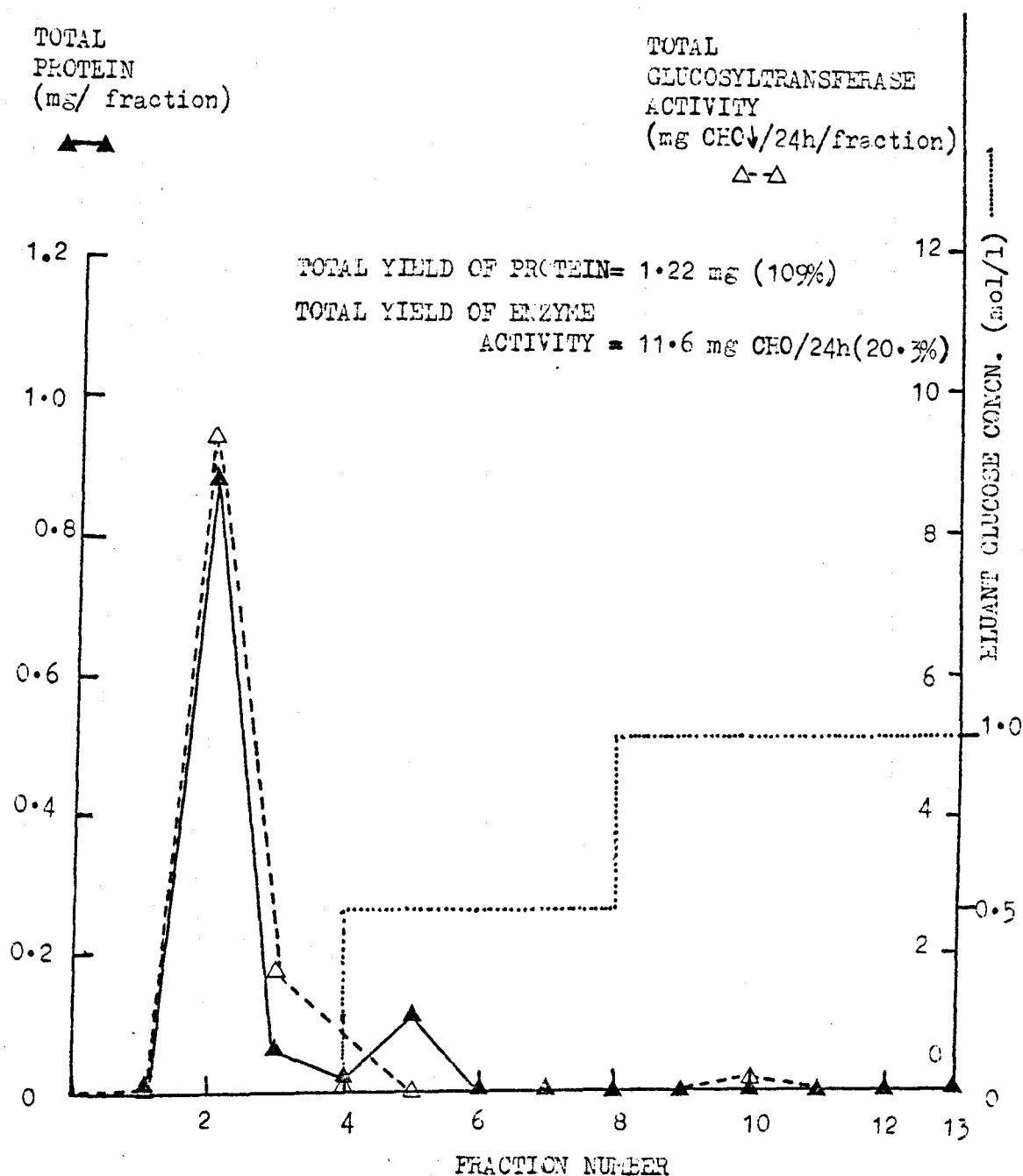
The glucosyltransferases of S. sanguis bind strongly to dextran and dextran-like glucans. As a result, the molecular weights of these enzymes cannot be determined by gel-filtration, since they bind to Sephadex (cross-linked dextran) and other gel-filtration media (Carlsson et al., 1969). However, this same property might be used for purification of the enzymes. For example, concanavalin A (Con A) binds

to dextran. Agrawal and Goldstein (1967) purified Con A from crude preparations of jack bean (Canavalia ensiformis) meal by binding it to columns of Sephadex G-50 and subsequently eluting it with 0.10 mmol/l glucose in 1.0 mol/l NaCl; Con A was also eluted from Sephadex G-50 by fructose, mannose, sucrose and methyl β -D-glucopyranoside. In this case, the column support material (Sephadex) was also the ligand for affinity chromatography.

Here, chromatography of ammonium sulphate purified glucosyltransferases on Sephadex G-10 as described in 2.3.4. (iv) was investigated. As can be seen, most of the enzyme activity which was applied remained bound to the column and could not be eluted by dextran or D-glucose, under the conditions used (see figs 3.6 and 3.7). Some enzyme was not bound and eluted with the void volume of the columns. It is possible that this was because the column was saturated with glucosyltransferase and no more could bind. However, hydroxylapatite chromatography (see fig. 3.4) of the same preparation showed that glucosyltransferases constitute only a small part of the total protein. These experiments show the same since almost all of the total protein was not bound to the column but most of the glucosyltransferase activity was bound. The total amount (dry weight) of Sephadex G-10 in a 10 ml column is about 4 g. The amount of glucosyltransferase is certainly much less than 1 mg (not allowing for any carbohydrate residues) since the sample contained 1.12 mg protein. Thus, the column was probably not saturated with bound protein, and the unbound glucosyltransferase activity may represent an enzyme (or enzymes) which cannot bind strongly to dextran.

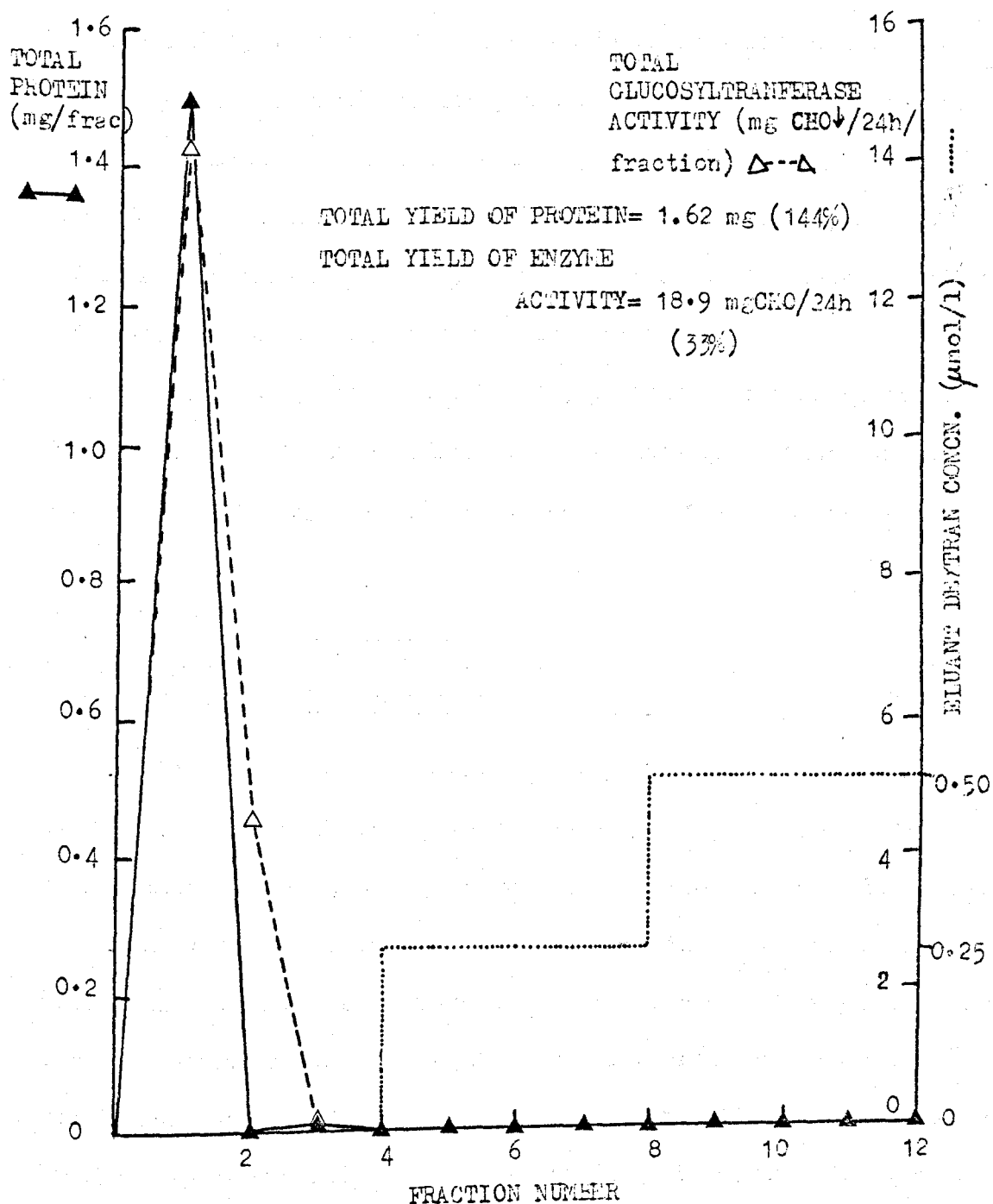
This was a preliminary study. Ideally, the experiments should

Fig. 3.6

AFFINITY CHROMATOGRAPHY OF $(\text{NH}_4)_2\text{SC}_4$ PURIFIEDGLUCOSYLTRANSFERASES ON SEPHADEX G-10 — ELUTION BY
GLUCOSENOTES

- (i) Sample volume = 1.0 ml. Total sample protein = 1.12 mg. Total sample glucosyltransferase activity = 57.1 mg CHO/24h.
- (ii) Flow rate = 0.23 ml/min. Fraction volume = 3.45 ml.
- (iii) Column bed volume = 9.5 ml (9.0 mm diam x 150 mm height). Void volume = 3.5 ml.

Fig. 3.7 AFFINITY CHROMATOGRAPHY OF $(\text{NH}_4)_2\text{SO}_4$ PURIFIED GLUCOSYL-
TRANSFERASES ON SEPHADEX G-10 — ELUTION BY DEXTAN T2000



NOTES

- (i) Sample volume= 1.0 ml. Total sample protein= 1.12 mg. Total sample glucosyltransferase activity= 57.1 mg CHO /24h.
- (ii) Flow rate= 0.24 ml/min. Fraction volume= 3.6 ml.
- (iii) Column bed volume= 9.5 ml (9.0 mm diam x 150 mm height).
Void volume= 3.0 ml.

be repeated with larger columns and smaller fraction sizes. However, it is unlikely that this technique could be used for purification of the enzymes. It would probably not be practical to use more drastic elution conditions. If, for example, the ionic strength of the eluant was increased, enzyme activity might be adversely affected.

If eluant dextran concentrations were increased, the background levels of precipitable glucan, in zero time controls for enzyme assays, would also be increased; even at 1.0 mg/l, dextran gave a high background. This would make it more difficult to estimate glucosyltransferase activity accurately, especially in fractions of low activity.

3.2.4. Effects of Metrizamide

(i) Reasons for investigating metrizamide

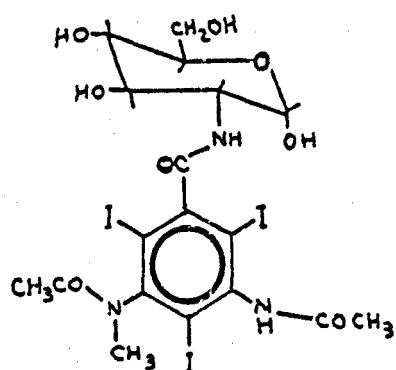
When studying any enzyme system, it is useful to know the molecular weights of the component enzymes. However, with the glucosyltransferases, there are some unique problems in mol.wt. determination by sedimentation velocity analysis in the ultracentrifuge. These enzymes cannot be run on sucrose gradients. The glucosyltransferases polymerise the glucosyl moiety of sucrose with no known requirements for co-factors. Even if such reactions could be minimised by low temperature and short spins, binding of enzyme molecules to the gradient medium (i.e. sucrose) would probably give an anomalous rate of sedimentation.

Glycerol gradients might be used but glycerol may inhibit or denature the glucosyltransferases of S. sanguis since some activity is lost after storage with glycerol at -20°C (J.A. Beeley and P.M. Black, unpublished observations).

There are many other density gradient media available. These media have hitherto only been used for equilibrium density gradient ultracentrifugation with macromolecules or for separation of cells or their organelles. However no one has yet considered the possibility of using these materials for mol.wt. estimation by sedimentation analysis: sucrose gradients were usually adequate for this purpose. The high ionic strength of the alkali metal salt gradients (such as CsCl, Cs₂SO₄ or organic salts of sodium or potassium) probably denature the glucosyltransferases and enzyme activity would be lost. With the various carbohydrates gradient media (Sorbitol, Ficoll) binding of enzyme to these compounds may give anomalous results; the glucosyltransferases of S. sanguis bind to various carbohydrates including cross-linked dextran (Sephadex) and agarose (Sephacrose) (Carlsson et al., 1969). The effects (and possible usefulness) of bovine serum albumin and 'Ludox' (colloidal silica preparation; Du Pont), which have been used as gradient media, are difficult to predict.

There are various iodinated compounds (e.g. metrizamide, sodium metrizoate, meglumine, urographin) being used, singly or in combinations, as ultracentrifuge density gradient media. These were originally used as contrast media for angiography and urography and so are generally considered to be biologically inert. Metrizamide [2-(3-acetamido-5-N-methyacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose; see fig 3.8] is a non-ionic medium widely used for equilibrium centrifugation. However, metrizamide solutions can reach the same densities and viscosities as sucrose solutions (information supplied by Nyegaard and Co., A.S., Oslo, Norway). Thus, it may be suitable for separation of proteins by sedimentation velocity techniques. The effects of metrizamide on the various methods of analysis used in this study were therefore studied.

Fig. 3.8 METRIZAMIDE



MOL.WT. 789.105

λ_{\max} 242 nm

$\epsilon_{242}^{25^\circ\text{C}}$ $3.3 \times 10^4 \text{ l. mol}^{-1} \text{ cm}^{-1}$

(ii) Effect of metrizamide on the Lowry method of protein assay

Various concentrations of metrizamide were added to the incubation mixture for the Lowry assay, in the absence of protein, and with two different concentrations of bovine α -chymotrypsinogen. Metrizamide reacted strongly with the Folin-Ciocalteu reagent giving a colour and extinction similar to that produced by equal weights of α -chymotrypsinogen (fig. 3.9). Metrizamide did not affect the reaction of protein with the reagent, but produced a high background extinction.

(iii) Effect of metrizamide on the phenol-H₂SO₄ assay for carbohydrate

Metrizamide is a derivative of 2-deoxy-D-glucose and might be expected to interfere with carbohydrate assays, such as the phenol-H₂SO₄ assay used in this study, in estimating glucosyltransferase activity.

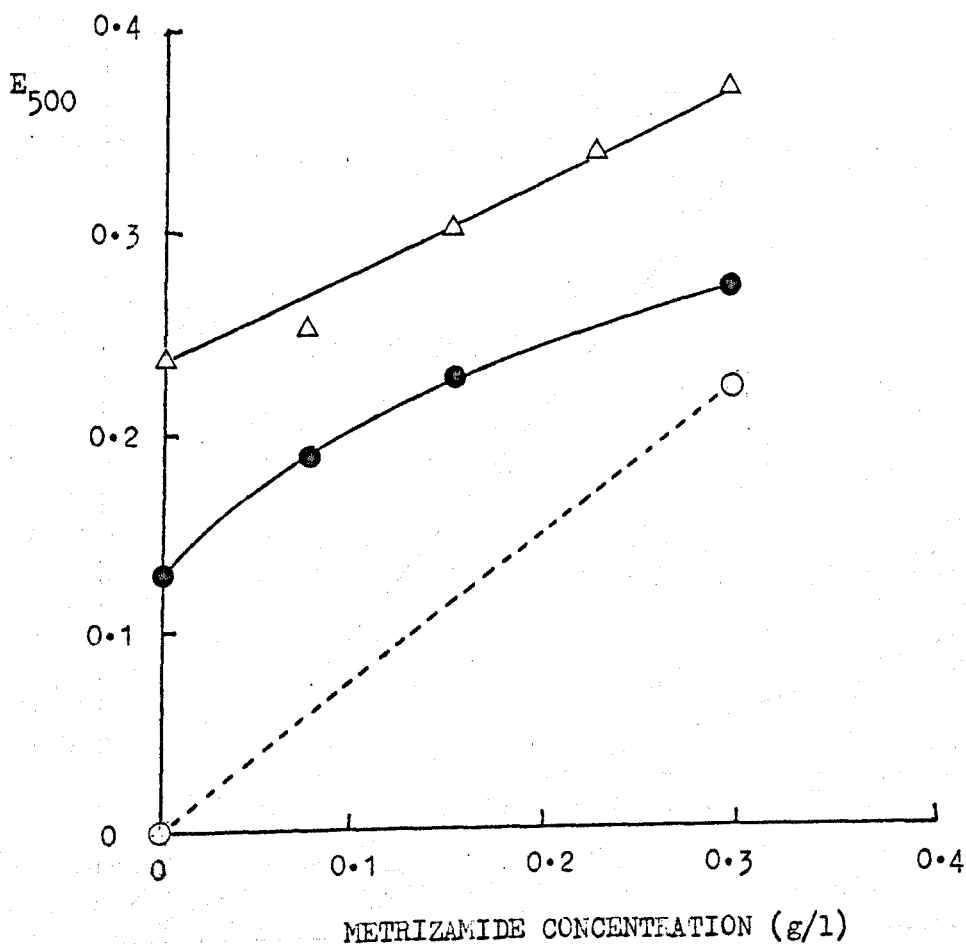
Metrizamide reacted strongly in the phenol-H₂SO₄ assay (see fig. 3.10). The colour produced was different from that produced by glucose; this is shown by the visible spectra of the two (see fig. 3.11). λ_{max} was 455 nm, as compared with 480-490 nm for many other sugars (Dubois et al., 1956). Even at 455 nm, however, the extinction was less than that produced by glucose.

However, this effect may not be a problem since no metrizamide, as measured by extinction at 242 nm (see fig. 3.12) was precipitated by sodium acetate and ethanol, even in the presence of the glucan and culture supernatant of S. sanguis 804. This confirms the finding of Mullock and Hinton (1973) that metrizamide is not precipitated by ethanol. Indeed, Monahan and Hall (1975) have separated chromatin from metrizamide, in fractions from density gradients, by precipitating the chromatin with sodium acetate and ethanol.

Fig. 3.9 EFFECT OF METRIZAMIDE ON THE LOWRY PROTEIN ASSAY

KEY

- Δ — Δ 0.185 g/l bovine α -chymotrypsinogen (0.074 mg/assay),
 \bullet — \bullet 0.093 g/l " " (0.037 mg/assay) ,
 \circ --- \circ 0 g/l " "



NOTES

- (i) The concentrations of metrizamide and chymotrypsinogen are those in the sample volume (0.4 ml) not the total assay volume (6.5 ml).

Figure 3.10

EFFECT OF METRIZAMIDE ON THE PHENOL-SULPHURIC ACID
ASSAY FOR CARBOHYDRATE

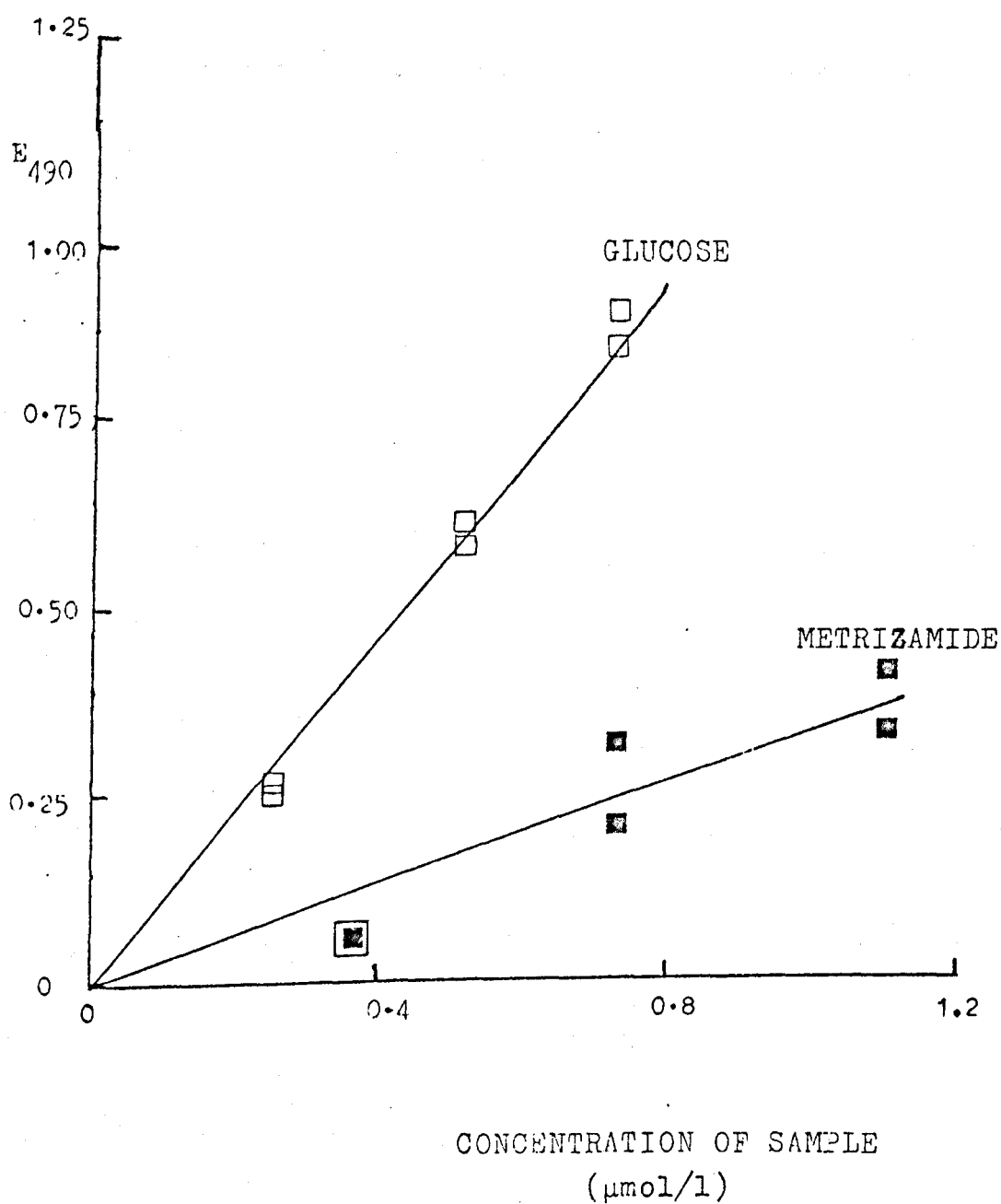


Fig. 3.11 VISIBLE SPECTRUM OF COLOUR PRODUCED IN PHENOL-H₂SO₄

ASSAY

NOTES

- (i) A Metrizamide concentration= $1.42 \mu\text{mol/assay}$.
B Glucose concentration= $0.253 \mu\text{mol/assay}$.
- (ii) Spectra were scanned on a Pye-Unicam SP800 spectrophotometer.

Figure 3.11 VISIBLE SPECTRUM OF COLOUR PRODUCED IN PHENOL- H_2SO_4 ASSAY

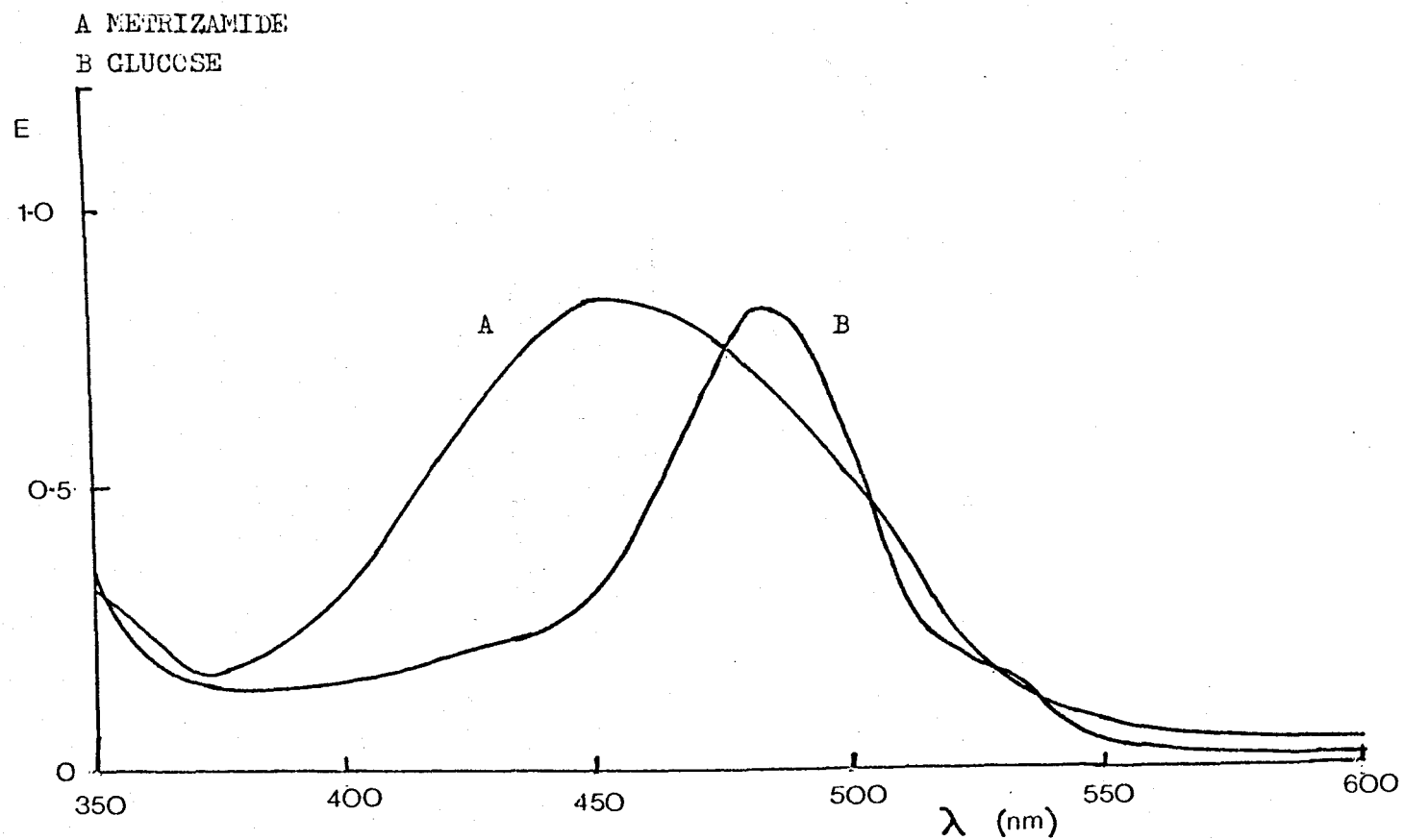
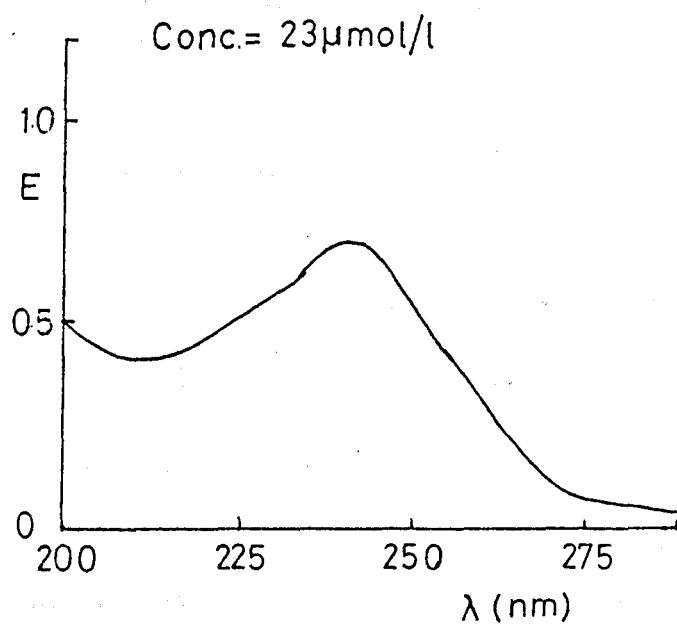


Fig. 3.12 UV Absorption spectrum of metrizamide in H₂O



(The spectrum was scanned using a Pye-Unicam SP800 spectrophotometer).

Metrizamide absorbs UV light strongly at 242 nm (see fig. 3.12). However, this property could not be used for estimating metrizamide in gradient fractions because of interference by protein and nucleic acid from the samples used. The method of Dubois et al. (1956) might well be useful for measuring concentrations of metrizamide; this would permit precise determination of the shapes of ultracentrifuge gradients. However, this would have to be studied further and the assay might be modified to minimise interference, especially from other carbohydrates.

(iv) Effect of metrizamide on glucosyltransferase activity

The enzyme used was culture supernatant from phase III of glucosyltransferase activity (32.5 h growth of S. sanguis 804) as described by Beeley and Black (1977).

Glucosyltransferase activity was strongly inhibited by metrizamide, reaching almost 80% inhibition by a metrizamide concentration of 170 $\mu\text{mol/l}$ (fig. 3.13). Without repeating this experiment at different concentrations of substrate (sucrose), one cannot determine the type of inhibition or the value of inhibitor constant (K_i).

It is not known whether or not this effect is reversible. Even if this were the case, it would be too time-consuming to remove metrizamide (e.g. by dialysis) from each of a large number of fractions from an ultracentrifuge gradient. Lengthy dialyses would probably also result in considerable losses of enzyme activity.

(v) Conclusions

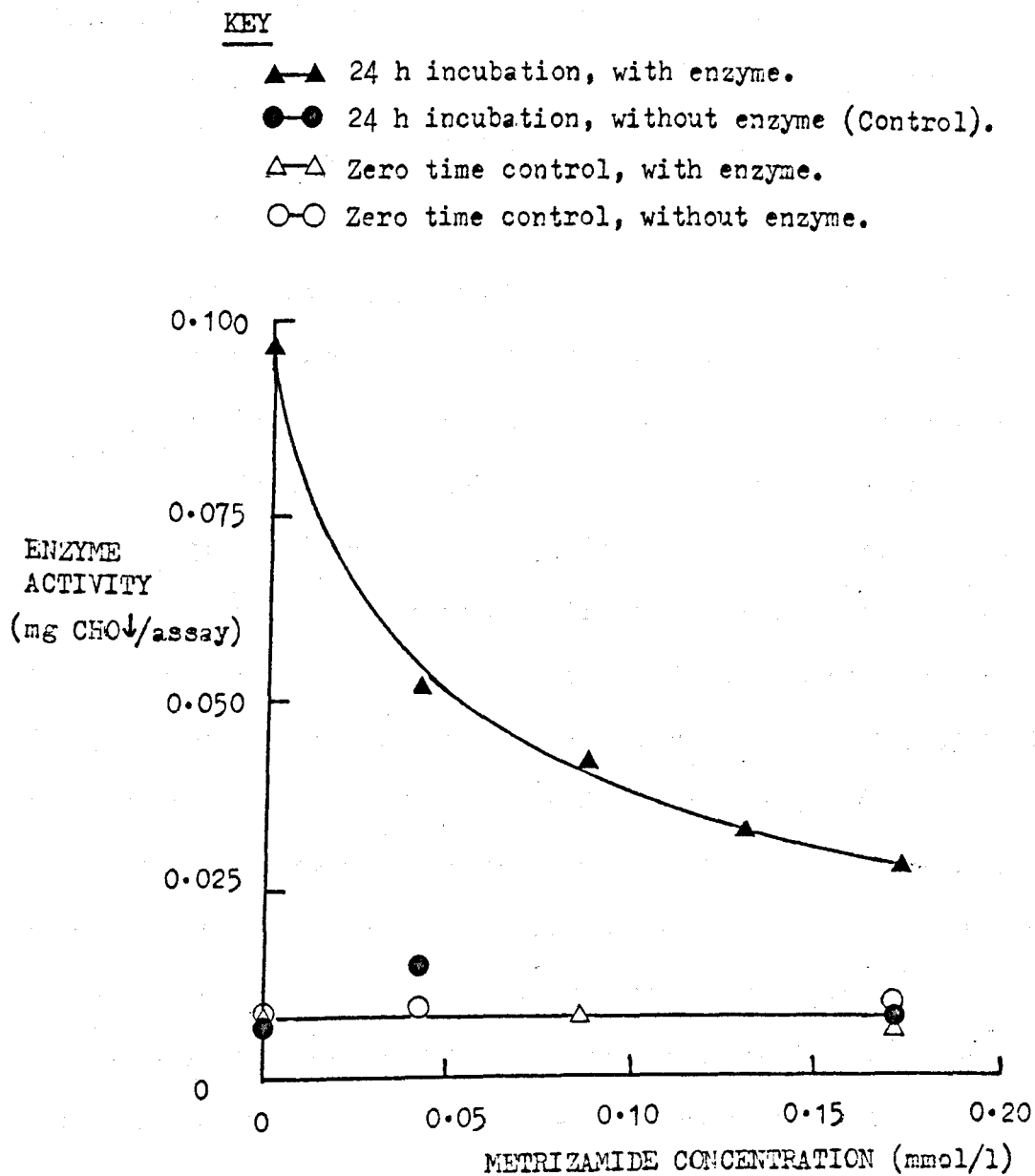
There appear to be too many practical difficulties involved for metrizamide to be of use in studying glucosyltransferases. For this reason, the use of metrizamide was not investigated further.

Fig. 3.13 Effect of metrizamide on glucosyltransferase activity

Enzyme activity was assayed by the method of Cybulska and Pakula (1963a) as modified by Beeley and Black (1977). All assays contained 0.2 ml of phase III culture supernatant (see text; $17\mu\text{g}$ protein/assay) in a total volume of 1.0 ml. Glucans were resuspended in 1.0 ml of NaOH solution (0.1 mol/l) and 0.5 ml was used in each carbohydrate assay.

The graph shows the effect of metrizamide on production of precipitable glucan in a 24 h incubation (open squares). The other three plots are controls for absence of enzyme, zero incubation, and both.

Fig. 3.13 Effect of metrizamide on glucosyltransferase activity



NOTES

- (i) Sucrose concentration= 147 mmol/l. pH= 7.0.
- (ii) Total amount of protein/ assay= 17.4 μ g.

3.3 Properties of Glucosyltransferase Preparations

3.3.1 Time course of enzyme activity

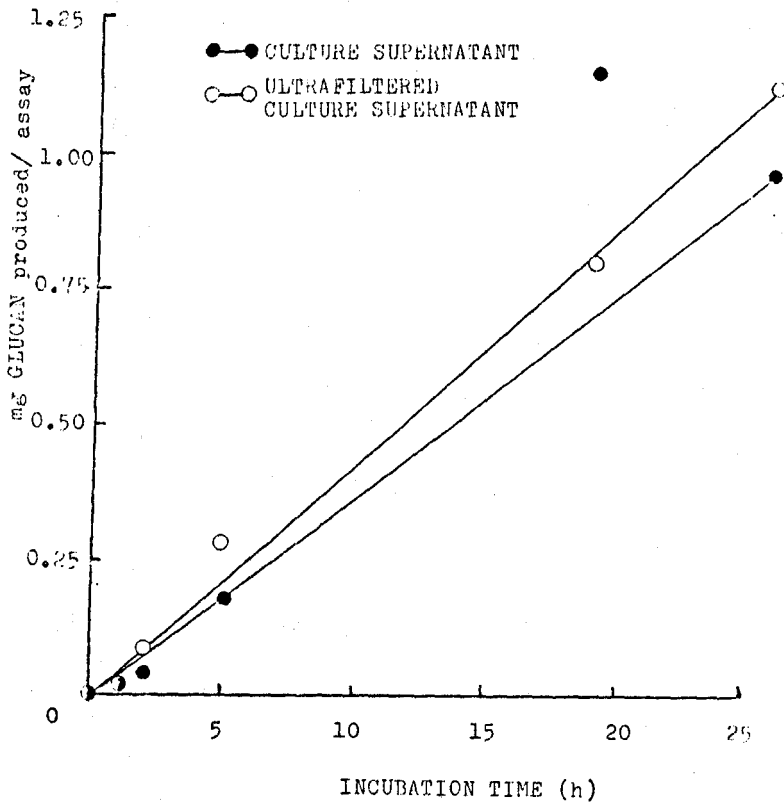
Black (1975) showed that production of ethanol-precipitable glucan from sucrose, by S. sanguis 804 culture supernatant was linear over 24 h. Production of reducing sugar (as fructose) was irregular, as measured by the method of Somogyi (1945), probably because of interference by oxidising and reducing compounds present in the crude supernatant.

In this study, glucan production was linear over 26 h with both crude and partially-purified preparations (figs 3.14 and 3.15). All these glucosyltransferase preparations can therefore be satisfactorily assayed by this method. Release of reducing sugar was linear only for $(\text{NH}_4)_2\text{SO}_4$ purified enzyme (fig. 3.16). The time course with ultra-filtered culture supernatant was slightly curved and that of untreated culture supernatant showed the same irregularity found by Black (1975). Assay by release of fructose by the method of Somogyi (1945) is thus suitable only for $(\text{NH}_4)_2\text{SO}_4$ purified preparations. (However, more exhaustive dialysis of the ultrafiltered culture supernatant might remove any interfering compounds.) An enzyme-linked assay for release of fructose might be useful, even for crude preparations. Unlike the Somogyi method, this would be much more specific for fructose. Newbrun and Carlsson (1969) used an enzyme-linked assay involving hexokinase, ATP, phosphoglucisomerase, glucose-6-phosphate dehydrogenase and NADP. Release of fructose was proportional to reduction of NADP (measured as ΔE_{340}). Glucose would also be detected by this method but this would not be a problem because the invertase of S. sanguis 804 is inducible by sucrose (McCabe et al., 1973) and would not be present in glucose-grown cultures. However, in this study, it did not prove necessary to

Fig. 3.14

TIME COURSE OF PRODUCTION OF PRECIPITABLE GLUCAN

BY *S.sanguis* 804 GLUCOSYLTRANSFERASES



NOTES

(i) Sucrose conc.= 147 mmol/l. pH= 7.0.

(ii) Total amount of protein/ assay;

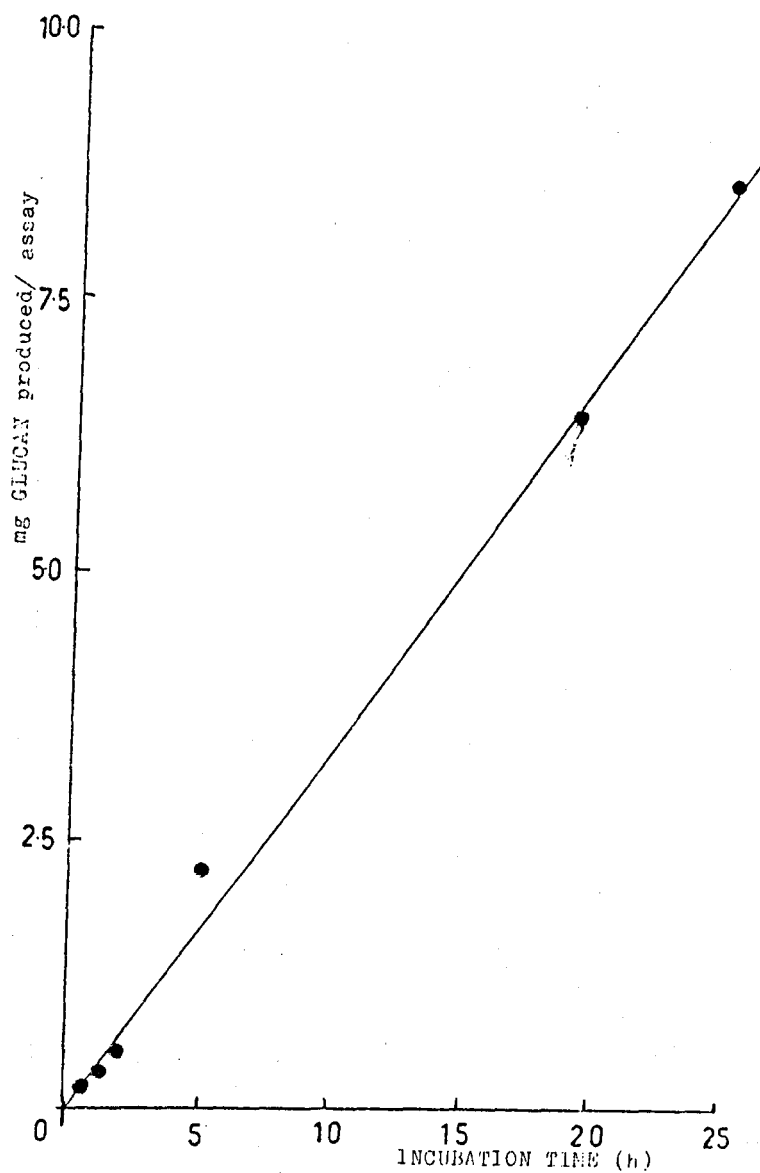
Culture supernatant 0.94 mg

Ultrafiltered

culture supernatant 0.40 mg

Fig. 3.15

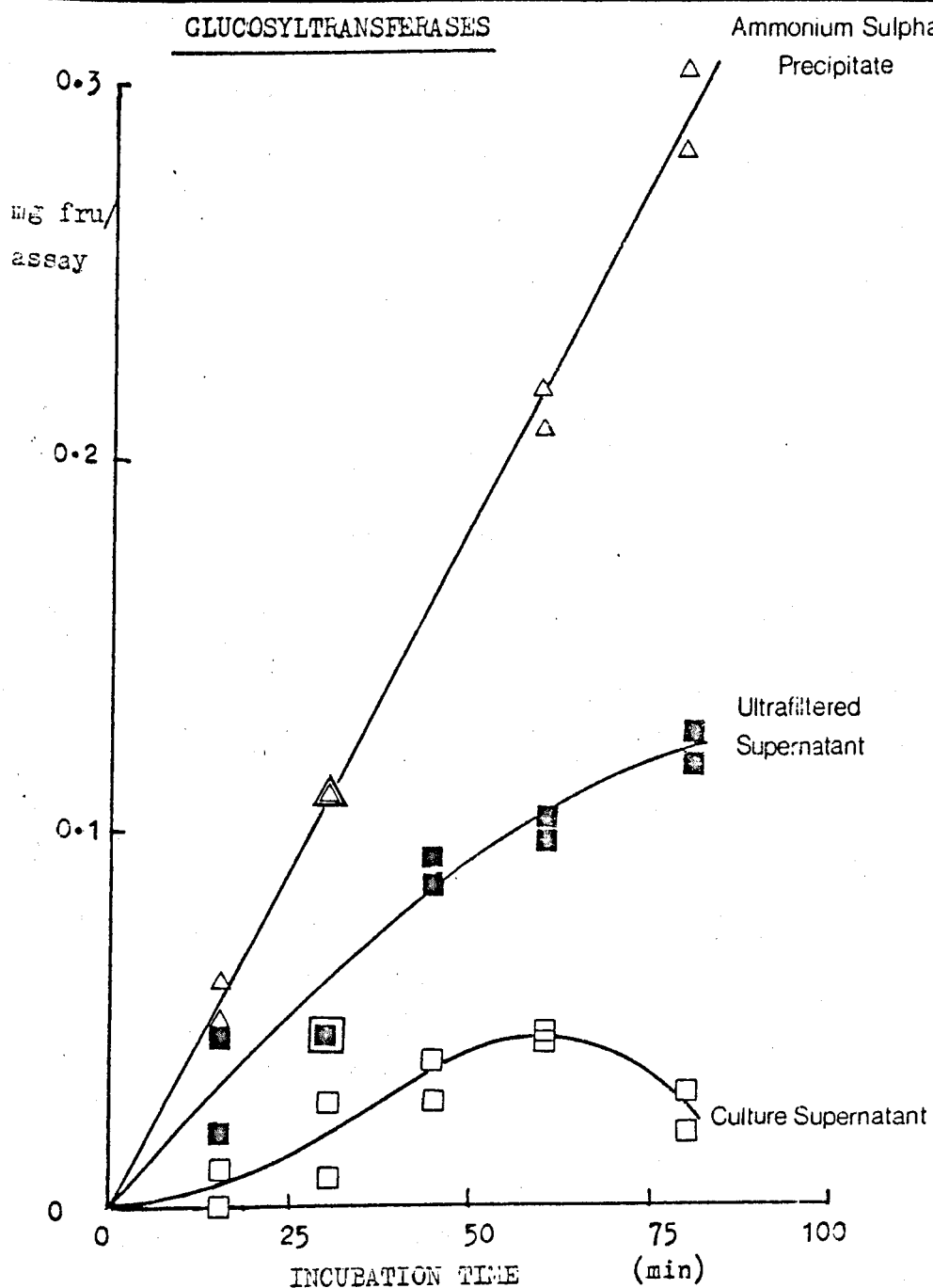
TIME COURSE OF PRODUCTION OF PRECIPITABLE GLUCAN BY
 $(\text{NH}_4)_2\text{SO}_4$ -PURIFIED GLUCOSYLTRANSFERASES OF *S.sanguis* 804



NOTES

- (i) Sucrose conc.= 147 mmol/l. pH= 7.0.
- (ii) Total amount of protein/ assay= 0.051 mg.

Fig 3.16 TIME COURSE OF RELEASE OF REDUCING POWER BY S. sanguis 804



NOTES

- (i) Sucrose concentration= 125 mmol/l. pH= 6.8.
- (ii) Assay incubation volume= 2.0 ml. Total amounts of protein per assay were as follows;

Culture supernatant	4.66 mg,
Ultrafiltered supernatant	1.99 mg,
Ammonium sulphate precipitate	0.103 mg.

develop an enzyme-coupled assay. In most experiments, the properties of the glucosyltransferases were studied using the ammonium sulphate purified preparations. Thus, the method of Carlsson (1969) was perfectly adequate. On a practical level, the reagents required for the Carlsson method, including those for the Somogyi assay, are cheaper, more easily prepared and more stable than the enzymes and other reagents for an enzyme-linked assay.

Hydroxylapatite-purified enzymes may have different time-courses because primers or acceptors would be removed by the purification. This was not investigated in this study but Germaine et al. (1974) showed that when hydroxylapatite purified glucosyltransferases of S. mutans were incubated with sucrose there was a lag before precipitable (i.e. high molecular weight) glucan was detectable. Sucrose and the newly-synthesised oligosaccharides were the only glucosyl acceptors present at first; some time was required for long glucan chains to be built up. This presumably only affects glucosyltransferase activity when measured as glucan synthesis. If enzyme activity is measured as release of fructose, the removal of acceptors by hydroxylapatite chromatography may have little effect.

3.3.2 Enzyme activity measured by different techniques

Glucosyltransferase activities were measured as synthesis of ethanol/sodium acetate precipitable polysaccharide (Cybulska & Pakula, 1963a; Beeley & Black, 1977) or as release of reducing sugar (Carlsson et al., 1969). The former method detects only those products which are of a sufficiently high molecular weight to be ethanol-precipitable. The smaller isomaltodextrins are not precipitated by ethanol. The latter technique, however, detects all glucosyl transfers from sucrose, regardless of the molecular weight or structure of the products.

Table 3.5 shows the specific activities of glucosyltransferase preparations at different stages of purification. In each case, the number of moles of fructose released is greater than the number of moles of glucose polymerised to precipitable glucan. This difference appeared to increase with purification, although, in other experiments, the difference found with the ammonium sulphate preparations was not always so marked. However, one would expect some difference with more purified enzyme since many trace glucosyl acceptors for high molecular weight glucan synthesis would be removed by purification; as explained already (Section 1.3.4), sucrose would then be the major acceptor and would lead to greater amounts of unprecipitable oligosaccharides.

Table 3.5

COMPARISON OF GLUCOSYLTRANSFERASE ASSAY TECHNIQUES

PREPARATION	GLUCOSYLTRANSFERASE ACTIVITY		RATIO	Fructose
	$\mu\text{mol PRODUCT/h/mg protein}$			Poly-
	Precipitable (1) Polysaccharide assay	Fructose assay		saccharide
Culture Supernatant	0.213	0.285 ⁽²⁾	1.34	
Ultrafiltered Culture Supernatant	0.649	1.45 ⁽²⁾	2.24	
0-70%(NH ₄) ₂ SO ₄ Enzyme	41.1	117	2.84	

Notes

1. Amount of polysaccharide is expressed in μmol of glucose.
2. Because of the irregular time course with these preparations, the figures given are estimates but are only correct to within 10%.
3. Sucrose concentration = 125 mmol/l. pH = 6.8.

3.4 Glucosyl Acceptors

3.4.1 Dextran T2000

High molecular weight dextrans are known to stimulate synthesis of glucans by extracellular glucosyltransferases of plaque streptococci (see section 1.3.4). Glucosyltransferase preparations in which high molecular weight glucan synthesis is only slightly stimulated by added dextran (Newbrun & Carlsson, 1969; Fukui *et al.*, 1974; Kuramitsu, 1975; Klein *et al.*, 1976) are probably not completely purified. Preparations free of all trace acceptors should, within limits, have an absolute requirement for added dextran (or other glucosyl acceptor). These 'limits' are the times required for synthesis of extended glucan chains, starting with sucrose as the initial glucosyl acceptor (Germaine *et al.*, 1974).

The effect of T2000 Dextran (mol.wt. 2×10^6 ; Pharmacia Fine Chemicals) on the activity of glucosyltransferases of *S. sanguis* 804 was investigated. Table 3.6 shows the effect of T2000 on different preparations of enzyme. Synthesis of precipitable glucan was stimulated at all degrees of purification examined. The most highly purified preparation studied (ammonium sulphate precipitated enzyme) was not stimulated any more than crude preparations (culture supernatant). This suggests that the ammonium sulphate preparations still contain many glucosyl acceptors, which may have remained bound to enzyme molecules during precipitation by ammonium sulphate.

Glucan synthesis by ammonium sulphate-purified glucosyltransferases was stimulated to about the same degree (about $2\frac{1}{2}$ -fold) by different concentrations of T2000, from 0.125 to 0.50 $\mu\text{mol/l}$ (see fig. 3.17). Thus, these concentrations must be nearly saturating,

Table 3.6

EFFECT OF T2000 DEXTRAN ON GLUCOSYLTRANSFERASE ACTIVITIES

ENZYME PREPARATION	ENZYME VELOCITY (mg CHO / h / mg protein)		DEGREE OF STIMULATION BY DEXTRAN [(b)/(a)]
	(a) CONTROL (no dextran)	(b) + DEXTRAN	
Culture supernatant	0.037	0.185	5.0
Ultrafiltered culture supernatant	0.112	0.306	2.7
Ammonium sulphate purified enzyme	6.94	23.9	3.4

NOTES

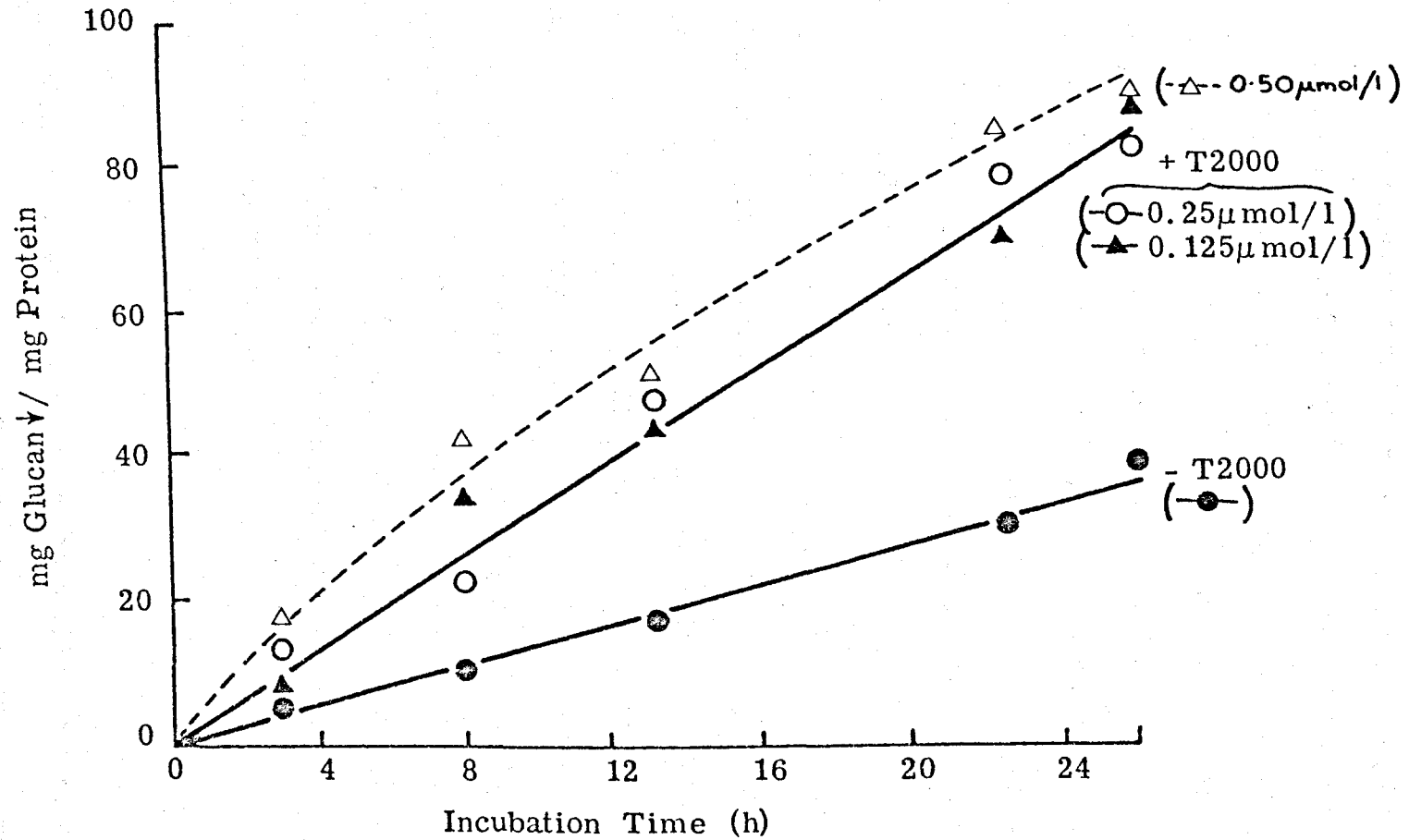
- (i) Sucrose concentration = 147 mmol/l. pH = 7.0.
- (ii) Dextran was T2000 (Pharmacia; mol.wt. 2×10^6). Dextran concentration was 0.25 μ mol/l (0.5 mg/ml).
- (iii) At the highest level of enzyme activity (ammonium sulphate purified enzyme + Dextran) the reaction velocity decreased over 24 h, possibly due to substrate exhaustion, therefore enzyme activities are expressed as initial velocities.

Fig. 3.17 Effect of T2000 Dextran on glucosyltransferase activity
of *S. sanguis* 804

NOTES

- (i) pH = 7.0.
- (ii) Assay incubation volume = 1.0 ml. Total amount of protein per assay = 35.7 μ g. Enzyme was ammonium sulphate purified preparation.
- (iii) Dextran was Dextran T2000 (Pharmacia; mol. wt. average 2×10^6).

Fig. 3.17 EFFECT of T2000 DEXTRAN on GLUCOSYLTRANSFERASE ACTIVITY
of S. SANGUIS 804



under the conditions used, and the K_a (i.e. K_m for a glucosyl acceptor) for T2000 must be very much less than $0.125 \mu\text{mol/l}$. Klein et al. (1976) found that the glucosyltransferase of S. sanguis had K_a for dextran of mol.wt. 4×10^4 was $200 \mu\text{mol/l}$. The large difference between their findings and those reported here may be due to the difference in the molecular weights of the dextrans used. Although values of K_a are useful for comparing acceptor specificities of different enzymes, they are not necessarily an accurate measure of K_m . Each dextran molecule may have several different enzyme binding sites. The number of possible acceptor sites for branching enzymes will be proportional to the molecular weight of the dextran.

No attempt was made to determine the value of K_a for Dextran T2000 with this glucosyltransferase system. Values of K_a would not be meaningful unless determined for individual glucosyltransferases present after they had been isolated and purified free of trace acceptors by hydroxylapatite chromatography. Moreover, as explained above, the concentration of acceptor sites may be larger, by an unknown factor, than the concentration of dextran molecules. In view of this, the various oligosaccharide series (e.g. the nigerodextrins and isomaltodextrins) appear more attractive as model systems for studying acceptor requirements of glucosyltransferases.

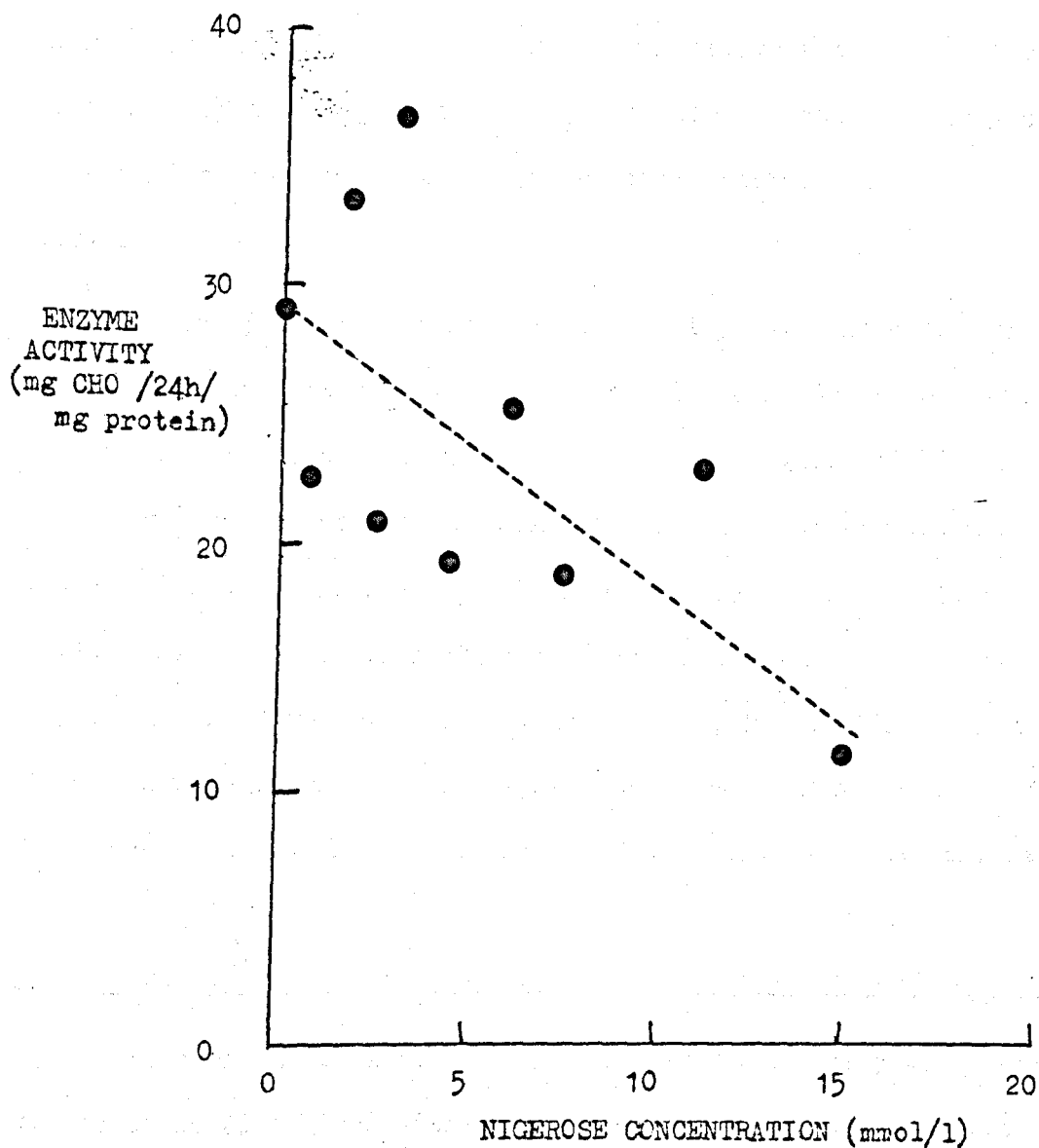
3.4.2 Nigerose

It has been shown (see 1.3.4) that various oligosaccharides stimulate the glucosyltransferases of S. sanguis and S. mutans, probably by acting as glucosyl acceptors.

One might expect nigerose ($\alpha\text{-D-gluc-1,3-}\alpha\text{-D-gluc}$) to be a

glucosyl acceptor, especially for 'mutansucrase' (see Appendix) enzymes. However, as shown by fig. 3.18, nigerose did not stimulate the synthesis of precipitable glucan by ammonium sulphate purified glucosyltransferases of S. sanguis 804. Glucan synthesis may have been slightly inhibited at the higher concentrations of nigerose, but this is not clear due to the scatter in the experimental points. Any inhibition would presumably be due to accumulation of short chain oligosaccharides arising from nigerose as acceptor. This also occurs when maltose is the added acceptor (Newbrun & Carlsson, 1969; Walker, 1972; see also 1.3.4 for discussion of this). It was not possible to investigate the effect of nigerose on glucosyltransferase activity as measured by release of fructose. The small amount of nigerose available was a gift from Dr. I.R. Johnson (Dept. of Biochemistry, University College, London) and most of this was used in the experiments described above. No further nigerose was available and the amounts of nigerotriose, nigerotetraose and nigeropentose obtained were too small for any experiments.

Fig. 3.18 EFFECT OF NIGEROSE ON GLUCOSYLTRANSFERASE ACTIVITY
OF *S. sanguis* 804



NOTES

- (i) Sucrose concentration= 147mmol/l. pH= 7.0.
- (ii) Assay incubation volume= 1.0 ml. .
- (iii) Total protein= 1.76 μ g/assay.
- (iv) The line plotted was calculated by linear regression.
 (R= 0.635). The great degree of scatter makes difficult to determine what the shape of the curve should actually be.

3.5 Kinetics of Glucosyltransferases

3.5.1 Effect of enzyme concentration on glucosyltransferases

The $(\text{NH}_4)_2\text{SO}_4$ preparations of glucosyltransferases described in section 3.2.1 are not completely purified and may contain trace inhibitors or activators of glucosyltransferase activity. Any effects of these might be expected to vary with concentration. It was thus important to determine how enzyme activity varied with concentration of enzyme.

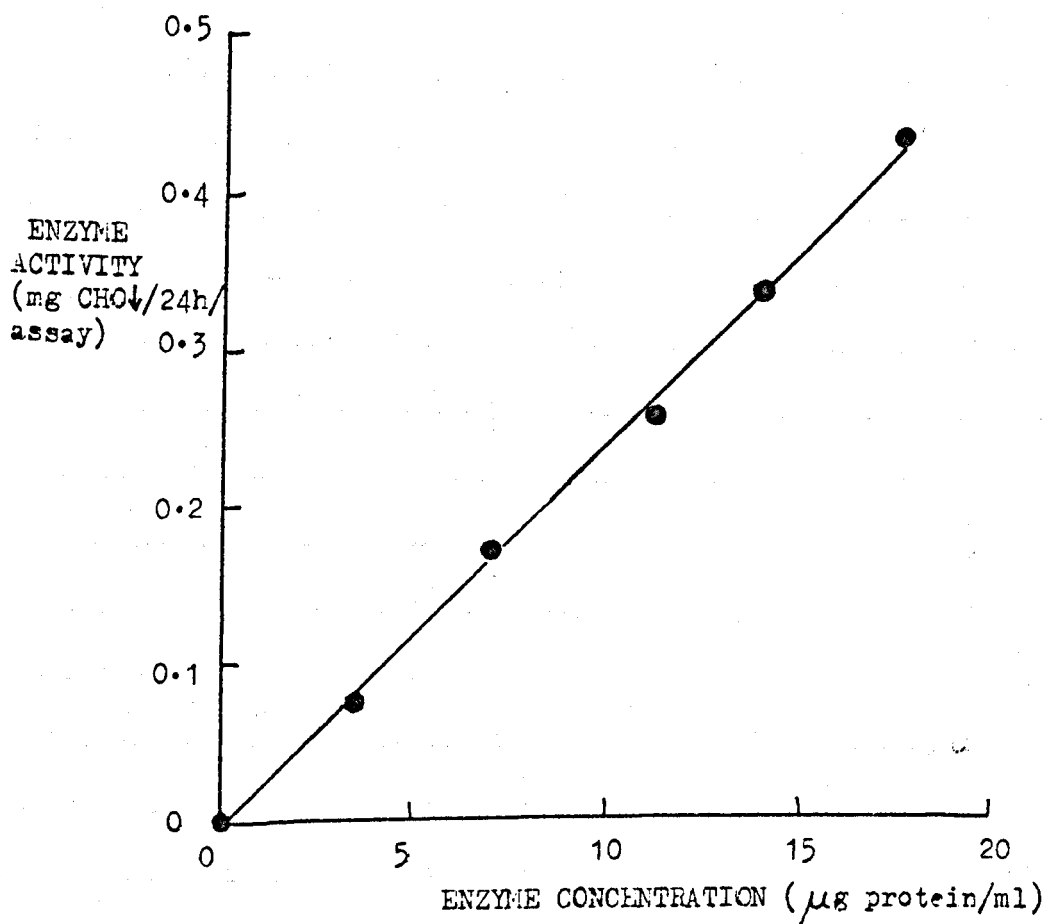
Fig. 3.19 shows that enzyme velocity, v , varied linearly with enzyme concentration, $[\text{E}]$. Therefore, the effects of contaminating activators or inhibitors, if present, are negligible. If there had been any significant effect, the plot of v against $[\text{E}]$ would have been curved. The interpretation of other kinetic studies would have been critically dependent upon the enzyme concentration used in the experiments. Since no effect was observed, it would seem that any convenient concentration of enzyme within the ranges shown in fig 3.19, can be used, depending on the needs of the particular experiment.

3.5.2 Effect of sucrose concentration on glucosyltransferase activity

The effect of sucrose concentration on activity of ammonium sulphate purified glucosyltransferases was studied using two assay techniques. One measured synthesis of precipitable glucan (Beeley & Black, 1977; modified from Cybulska & Pakula, 1963a) and the other measured release of fructose as reducing sugar in alkaline conditions (Carlsson, 1969). (For full details of both techniques, see 2.3.3.)

It has already been shown (see Section 3.3.2) that, at a sucrose concentration of 125 mmol/l, apparent enzyme activity was greater when

Fig. 3.19 EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF
 $(\text{NH}_4)_2\text{SO}_4$ PURIFIED GLUCOSYLTRANSFERASES



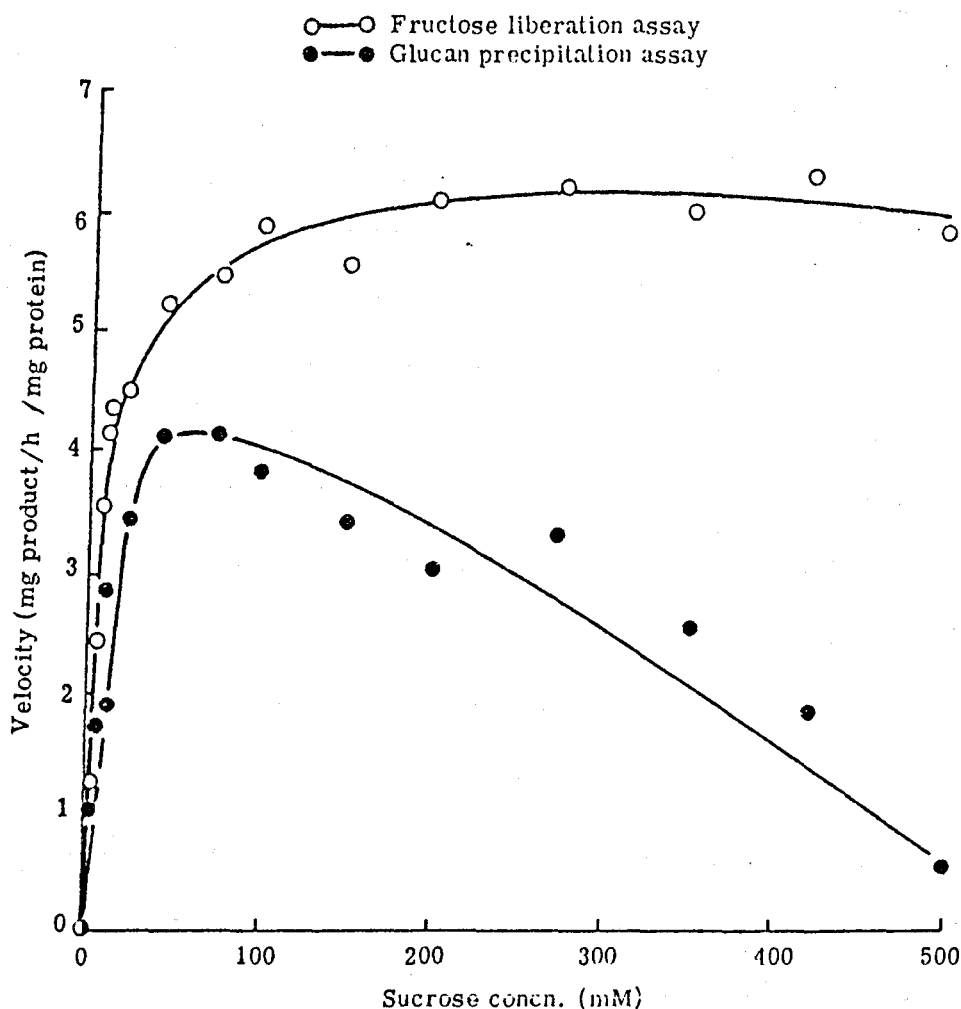
NOTES

- (i) Sucrose concentration= 147 mmol/l. pH= 7.0.
- (ii) Assay incubation volume= 1.0 ml.

measured as release of fructose than as synthesis of glucan. The results, shown in fig. 3.20, show that this is also the case at other concentrations of sucrose, up to 500 mM and that the ratio of the two (i.e. fructose release/glucan synthesis) is greater at higher concentrations of sucrose. This suggests that, at high concentrations of sucrose, a greater proportion of the reaction products are of low molecular weight and non-precipitable (i.e. oligosaccharides) rather than glucans.

It was also found (fig 3.20) that at very high concentrations of sucrose (up to 500 mmol/l), synthesis of precipitable glucans was substantially inhibited, although release of fructose was hardly affected. The rate of glucan synthesis increased with increasing sucrose concentration, up to about 80 mmol/l. Activity was maximal between about 70 to 100 mmol/l sucrose (although the precise maximum varied between preparations) but was inhibited at higher concentrations. At 500 mmol/l, the rate of glucan synthesis was only about 6% of the theoretical uninhibited V_{max} , as extrapolated from Lineweaver-Burk plots. In fact, at all sucrose concentrations above about 50 mmol/l, the rate of glucan synthesis was significantly less than the theoretical uninhibited values (see fig 3.21). This inhibition has important biological and clinical implications. The ability of many plaque bacteria to bind to the teeth is dependent on the synthesis of extra-cellular glucans from sucrose by S. sanguis and other microorganisms (see sections 1.2.3, 1.2.4 and 1.2.5). The rates of glucan synthesis in plaque and of plaque formation will thus be related to dietary and oral concentrations of sucrose. If these concentrations were ever high enough, glucan synthesis (and hence, plaque formation) might actually

Fig. 3.20 EFFECT OF SUCROSE CONCENTRATION ON GLUCOSYLTRANSFERASE ACTIVITY



NOTES

- (i) pH= 7.0.
- (ii) Assay incubation volume= 1.0 ml. Total amount of protein per assay= 30 μ g. Enzyme was $(\text{NH}_4)_2\text{SO}_4$ purified preparation.
- (iii) Triplicate zero-time and 24 h incubation tubes were set up for the glucan precipitation assay, as normal (see section 2.3.3). In addition, a seventh tube was set up for each sucrose concentration; at zero-time and after 2 h incubation, two 0.2 ml samples were withdrawn from these tubes and assayed for fructose concentration.

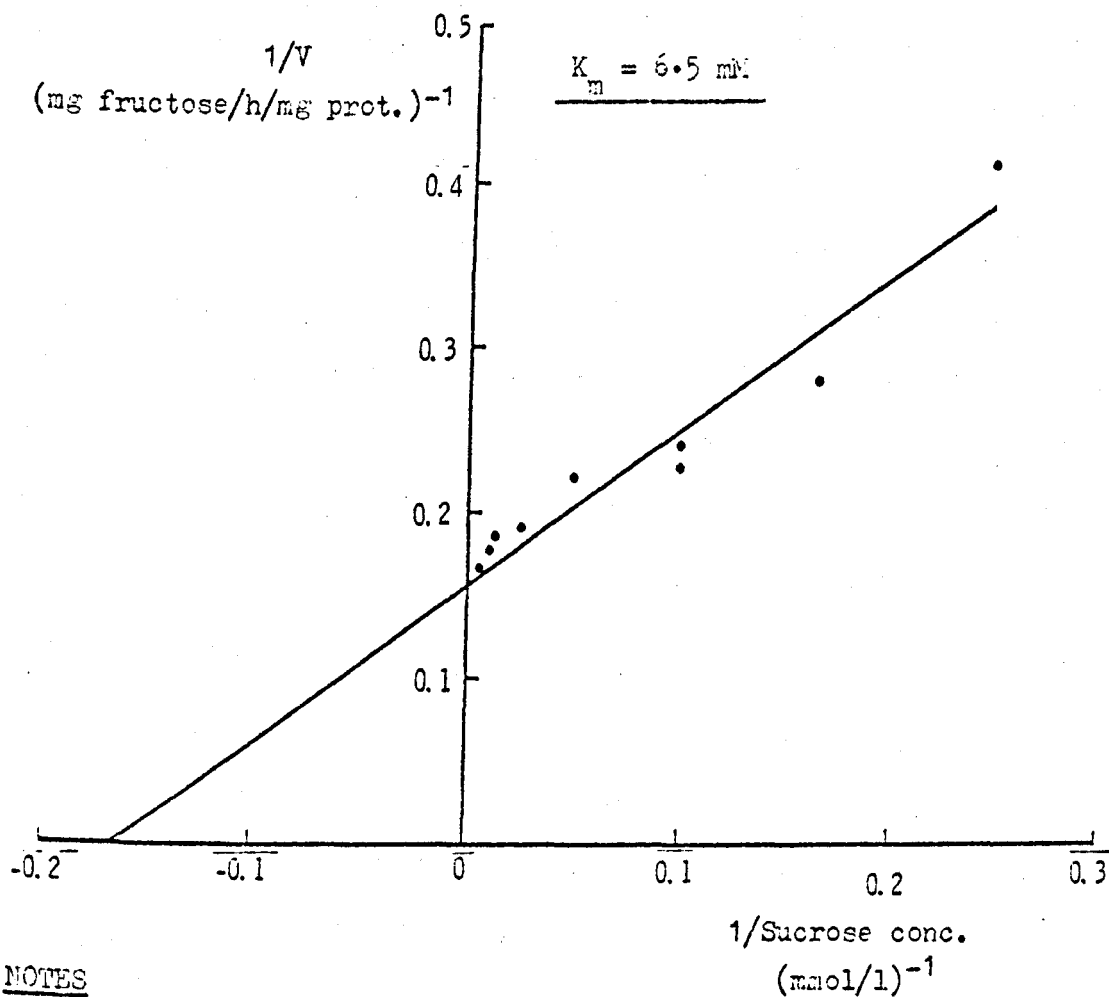
be inhibited, in vivo. For this reason, the project was extended to include studies of the concentrations of sucrose in various sweet foods and beverages, and in saliva and dental plaque (see section 3.7).

The rate of glucan synthesis, in vivo and in vitro, is also affected by the values of K_m , for sucrose, of the glucosyltransferases. The K_m for the ammonium sulphate purified enzymes was calculated using Lineweaver-Burk double-reciprocal plots ($\frac{1}{v}$ against $\frac{1}{[S]}$). This procedure is now largely disfavoured for determining values of K_m because it weights points at low substrate concentrations. These points, having low enzyme activities, are usually more prone to experimental error. However, in cases of substrate inhibition, the low concentrations are the most useful for determining K_m . This is because plots of $\frac{1}{v}$ against $\frac{1}{[S]}$ deviate from the linearity of Michaelis-Menten kinetics at high concentrations of substrate.

Admittedly, the ammonium sulphate preparations are not completely pure. They contain more than one enzyme and, possibly, some trace glucosyl acceptors. Thus, any values of K_m , reported in this study, are 'apparent K_m ' (K_m^{app}) and do not reflect the properties of the active site of any one particular enzyme. However, this K_m^{app} , for the ammonium sulphate preparations, will be a useful indication of the behaviour of the glucan-synthesising system, as a whole.

Different values of K_m were obtained with the two assay methods used. When enzyme activity was measured as release of fructose, the values of K_m obtained varied little between experiments. The mean value was 5.9 ± 1.1 (S.D.; $n = 4$) mmol/l. (See fig. 3.21). When activity was measured as rate of glucan synthesis, the measured values

Fig 3.21 LINEWEAVER-BURK PLOT FOR GLUCOSYLTRANSFERASE ACTIVITY -
- RELEASE OF FRUCTOSE



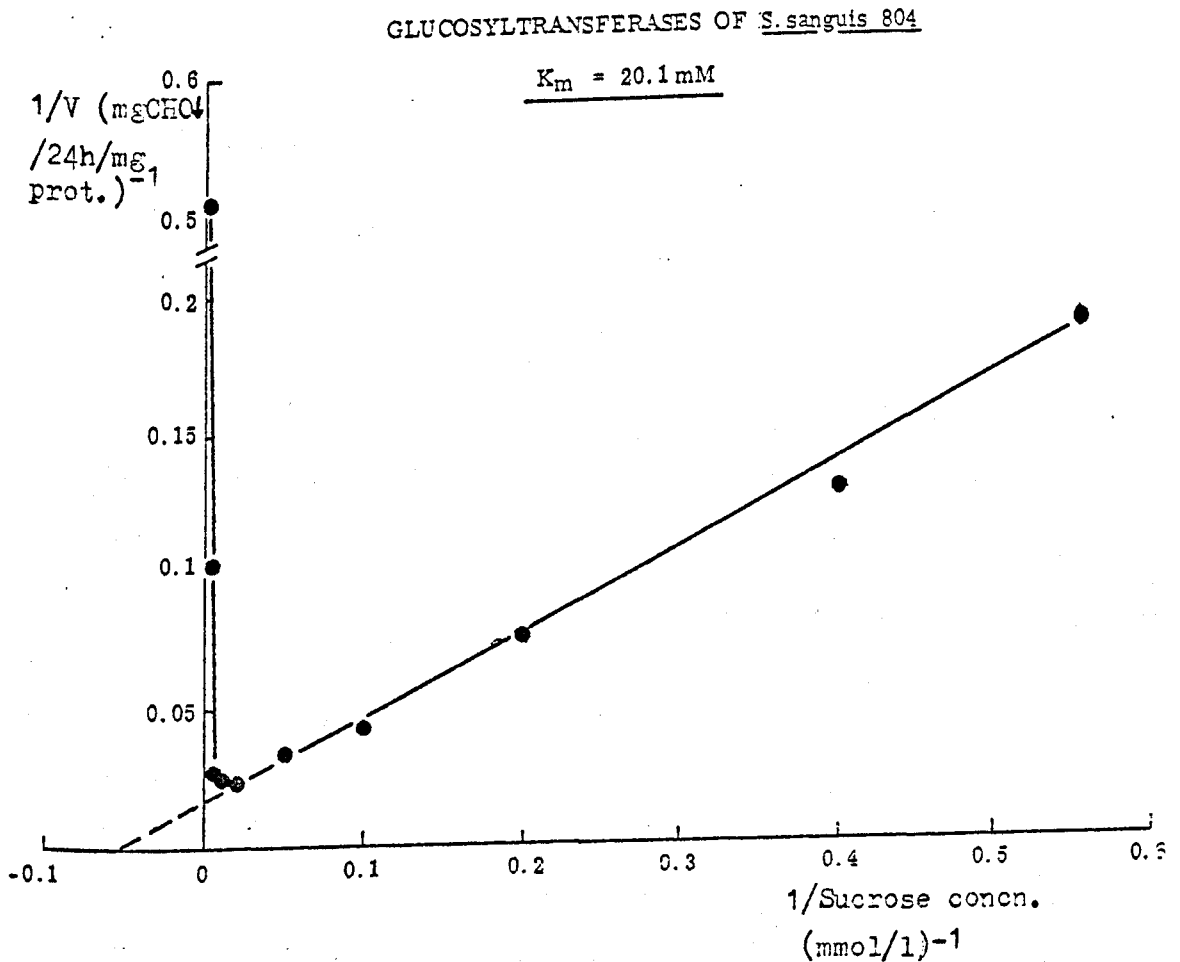
NOTES

- (i) pH= 7.0
- (ii) Assay incubation volume= 1.0 ml. Total amount of protein per assay was 30 μg . Enzyme was $(\text{NH}_4)_2\text{SO}_4$ purified preparation.

varied greatly (10.4-22.0 mmol/l) but most values were about 20 mmol/l (see fig. 3.22). The mean value of K_m was 17 ± 5 (S.D.; $n = 4$) mmol/l. It is not surprising that the two assay methods gave different values of K_m , since the ammonium sulphate preparations may contain several enzymes (see section 3.2.2) and, possibly, substantial amounts of glucosyl acceptor compounds. Thus, not all of the glucosyl transfers catalysed by the enzyme will lead immediately to precipitable glucan molecules. This argument leads to the result actually found here, that the apparent K_m for synthesis of precipitable glucan is higher than that for total glucosyl transfer (as measured by release of fructose).

As is shown below (section 3.7) the oral and dietary concentrations of sucrose very often exceeded the K_m for glucan synthesis, suggesting that these glucosyltransferases may often be very active in vivo (i.e. in dental plaque).

Fig. 3.22 LINWEAVER-BURK PLOT FOR GLUCOSYLTRANSFERASE ACTIVITY -
- PRODUCTION OF PRECIPITABLE GLUCAN



NOTES

- (i) pH = 7.0.
- (ii) Assay incubation volume = 1.0 ml. Total amount of protein per assay was $35.7 \mu\text{g}$. Enzyme was ammonium sulphate purified preparation.

3.6 Inhibitors of Glucosyltransferases

3.6.1 Introduction

Apart from scientific interest, there are important clinical reasons for investigating possible inhibitors of the extracellular glucosyltransferases of S. sanguis. It is now clear (see Sections 1.2.4 and 1.2.5) that synthesis of adhesive extracellular glucans by S. sanguis is important in early plaque formation and hence, ultimately, in cariogenesis. If an effective and reliable method of inhibiting the glucosyltransferases could be found, it might be useful in limiting plaque formation and might help eliminate dental caries. It would however be over-optimistic to expect that this approach alone could completely eliminate caries since some weakly cariogenic bacteria (e.g. S. sanguis and S. salivarius) can bind to the tooth surface without the aid of adhesive extracellular glucans (see 1.2.4).

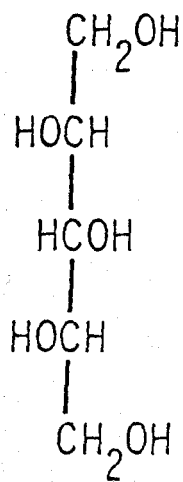
3.6.2 Xylitol

Xylitol (see fig 3.23) is an optically inactive pentitol currently being investigated as a possible dietary alternative to sucrose as the main sweetener in the diet.

Many workers have proposed that xylitol is anticariogenic, that is, that it actively inhibits dental caries. In particular, Knuuttila and Mäkinen (1975) found that the glucosyltransferases of S. mutans were stimulated by low concentration of xylitol (less than 400 mmol/l) but were inhibited by higher concentrations reaching about 35% inhibition by about 890 mmol/l xylitol.

The glucosyltransferases of S. sanguis are also important in cariogenesis so the effect of xylitol on the activity of the $(\text{NH}_4)_2\text{SO}_4$

Fig. 3.23 XYLITOL



purified enzymes was investigated. Over a large range of xylitol concentrations (0.01 - 900 mmol/l), xylitol neither stimulated nor inhibited enzyme activity (see table 3.7).

Thus, if xylitol does indeed inhibit cariogenesis or plaque formation, it is unlikely that it does so by inhibiting glucan synthesis during early plaque formation.

3.6.3 Hydrogen peroxide

S. sanguis has no catalase or peroxidase and, when grown aerobically, produces auto-inhibitory concentrations of hydrogen peroxide (Holmberg & Hallander, 1973; Donoghue, 1974). Beeley et al. (1976) found that growth and glucosyltransferase production by S. sanguis 804 are stimulated in mixed culture with catalase-producing bacteria such as Staph. aureus. A similar, although less, marked, effect was observed when sterile catalase was added to pure broth cultures of S. sanguis 804. Thus, it appeared that hydrogen peroxide might limit glucosyltransferase production. The possibility that hydrogen peroxide might have inhibited glucosyltransferase activity directly could not be dismissed, therefore this was investigated in this study.

The effect of various concentrations of hydrogen peroxide on the activity of ammonium sulphate purified glucosyltransferase as measured by release of fructose was examined. Fig. 3.24 is a Lineweaver-Burk plot of the results obtained. Hydrogen peroxide had no discernible effect at any concentration studied up to 1.2 mmol/l. The concentrations of hydrogen peroxide were much smaller than those of the fructose released from sucrose by the glucosyltransferases. Thus, it was extremely unlikely that hydrogen peroxide would interfere

Table 3.7

EFFECT OF XYLITOL ON $(\text{NH}_4)_2\text{SO}_4$ PURIFIED GLUCOSYLTRANSFERASES

XYLITOL (mmol/l)	GLUCOSYLTRANSFERASE ACTIVITY (mg fructose/h/mg protein)
0	5.1
0.01	5.5
0.9	5.0
10	5.5
100	4.9
600	5.0
900	5.1

NOTES

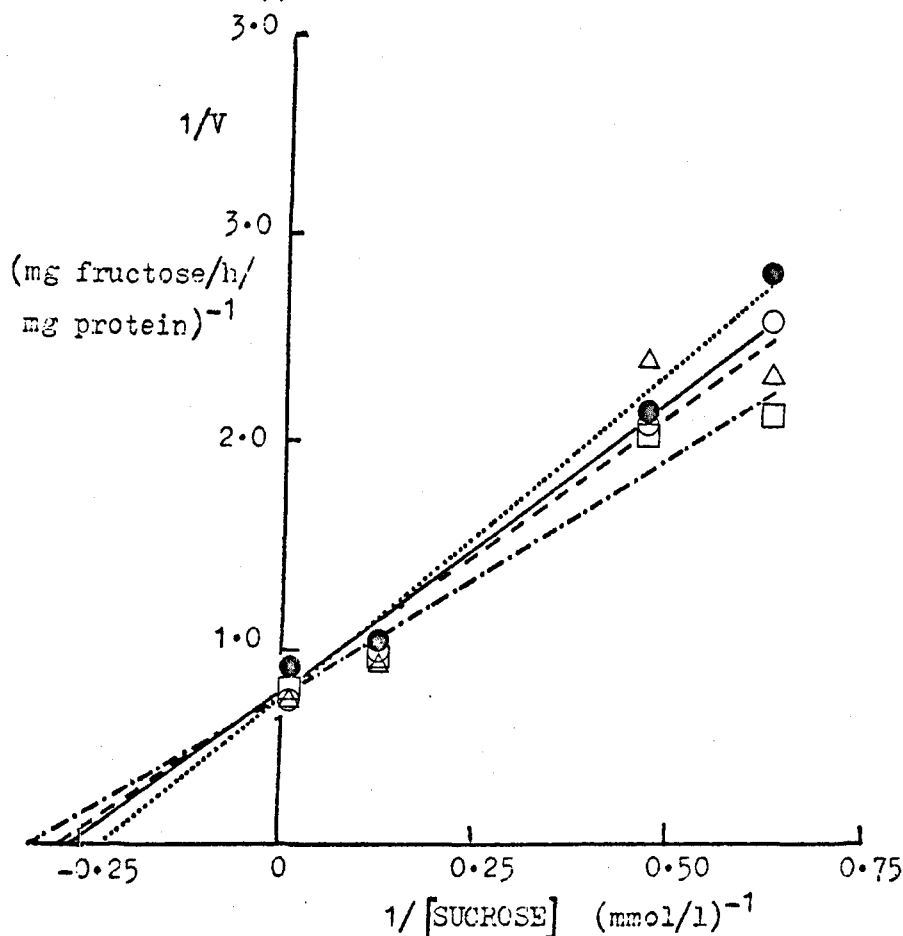
(i) Sucrose concentration = 125 mmol/l, pH = 6.8.

Fig. 3.24 EFFECT OF HYDROGEN PEROXIDE ON GLUCOSYLTRANSFERASE

ACTIVITY

KEY ○—○ CONTROL (no H_2O_2) □---□ $50.2 \mu\text{mol/l}$ H_2O_2
 △---△ $5.02 \mu\text{mol/l}$ H_2O_2 ●---● 1.04 mmol/l H_2O_2

(Other points, at different H_2O_2 concentrations, have been omitted for clarity).



NOTES

- (i) pH= 6.8.
- (ii) Assay incubation volume= 1.0 ml.
- (iii) Total amount of protein/ assay= $51.4 \mu\text{g.}[(\text{NH}_4)_2\text{SO}_4 \text{ preparation}]$.
- (iv) In control (no H_2O_2), $K_m^{\text{sucrose}} = 5.6 \text{ mmol/l}$.
- (v) All lines were calculated by linear regression and the regression coefficients were as follows;

Control, 0.99; 5.02 mol/l H_2O_2 , 0.98; 50.2 mol/l , 0.96;
 1.04 mmol/l , 0.99.

significantly with the reduction of Cu(III) to Cu(II) in the reducing sugar assay (Somogyi, 1945).

If the lower level of glucosyltransferase activity in pure cultures as compared with mixed cultures, is due to the presence of hydrogen peroxide, the effect is not due to inhibition of enzyme activity. Hydrogen peroxide presumably inhibits synthesis of the glucosyltransferases. This is probably not a specific effect but due to the general cytotoxicity of hydrogen peroxide.

3.7 Intra-oral and Dietary Concentrations of Sucrose

3.7.1 Introduction

The importance of sucrose metabolism in plaque formation and cariogenesis has already been discussed (section 1.2). Production of extracellular glucans by S. sanguis is important in early plaque formation and the effect of sucrose concentration on synthesis of these glucans has been investigated in this study (section 3.5.2).

Many other workers have investigated the metabolism of sucrose by dental plaque and the kinetics of sucrose-metabolising enzymes of plaque bacteria. Surprisingly, however, none have yet attempted to relate these studies to the concentrations of sucrose which occur in cariogenic foods, and in saliva and dental plaque. Hardinge et al. (1965) compiled extensive data on individual carbohydrate components (including sucrose) of many foodstuffs. However, these tables are of limited value since they include few of the most cariogenic components of the diet, such as hard and soft sweets and sweetened soft drinks. There have been some limited studies of the concentrations of 'sugar' and its clearance from the mouth. These only measured glucose or reducing sugar (Lanke, 1957; Adorjan & Stack, 1976). In the latter study, measurements were qualitative or, at best, semi-quantitative, using reagent strips (e.g. Ames 'Clinistix').

Sucrose is more difficult to assay than other sugars such as glucose and fructose. There is no simple or specific chemical assay for sucrose. It must be determined either as total carbohydrate after chromatographic separation or by enzyme-linked assay of glucose or hexose (i.e. glucose + fructose) after hydrolysis with invertase (Bergmeyer & Klotsch, 1963).

As shown earlier (3.5.2), partially purified glucosyltransferases of S. sanguis 804 are inhibited by high concentrations of sucrose. Some workers have also reported that acid production from sucrose, by salivary bacteria and dental plaque, is inhibited by high sucrose concentrations (Geddes, 1975 & 1977; Sandham & Kleinberg, 1969; Birkhed & Frostell, 1978). It was thus important to examine oral and dietary sucrose concentrations, during and after ingestion of sucrose-containing foods and drinks in order to get some idea of the activity of glucan-producing enzymes in plaque. This study was made much easier by a new commercial kit for estimation of sucrose (Boehringer Sucrose/Glucose UV Test Kit).

3.7.2 Concentrations of sucrose in sweet beverages

The sucrose concentrations in various beverages are listed in Table 3.8. Some of the concentrations were assayed directly. Others were calculated from the weight of sucrose used (sweetened tea or coffee) or calculated from published data. For the latter, water and total carbohydrate contents were obtained from 'Documenta Geigy' (Diem & Lentner, 1970; pp499-515). The sucrose contents were obtained from Hardinge et al. (1965). The concentrations of sucrose were then calculated on the assumption that the density of each beverage was about $1.0 \times 10^3 \text{ kg.m}^{-3}$. (Even at the comparatively high concentration of 500 mmol/l, the density of a sucrose solution is only $1.07 \times 10^3 \text{ kg.m}^{-3}$). The upper values given for 'Cola' drinks (other than 'Coca-Cola' and 'Pepsi-Cola') and carbonated soft drinks were calculated from 'Documenta Geigy' on the basis that all the sugar present is sucrose. The lower limits were added because much of the sucrose in proprietary beverages

Table 3.8 SUCROSE CONCENTRATIONS OF SOME SWEET BEVERAGES

BEVERAGE	SUCROSE CONCENTRATION ⁽ⁱ⁾ (mmol/l)	CALCULATED GLUCO- ⁽ⁱⁱ⁾ SYLTRANSFERASE ACTIVITY (%age of maximum)
Sweetened tea or coffee		
(A) 1 teaspoon (6 g)/150 ml	117 ⁽¹⁾	94
(B) 3 teaspoons (18 g)/150 ml	351 ⁽¹⁾	48
'Coca-Cola'	350 ⁽²⁾	48
'Pepsi-Cola'	323 ⁽²⁾	54
Other 'Cola' drinks	200-320 ^{(3),(5)}	55-80
Carbonated soft drinks	200-400 ^{(3),(5)}	37-80
Apple juice (fresh, unsweetened)	12 ⁽⁴⁾	67
Citrus juices (fresh, un- sweetened)	3-15 ⁽⁴⁾	18-75

NOTES

(i) Sucrose concentrations were determined as follows:

- (1) Calculated
- (2) Estimated by sucrose assay procedure (see section 2.3.3)
- (3) Calculated from data in Diem & Lentner (1970)
- (4) Calculated from data in Hardinge et al. (1969)
- (5) Allowance made for occasional addition of saccharin.

(ii) Interpolated from fig. 3.20.

(iii) The sucrose contents (as g/100 g) of many other foods and beverages have been compiled by Hardinge et al. (1965).

may be replaced by saccharin when the market price of sucrose is high (information supplied by J. Garvie and Sons Ltd., Milngavie, Glasgow, Scotland).

Table 3.8 also shows the relative (%age) activity, at these sucrose concentrations, of the ammonium sulphate purified glucosyltransferases (interpolated from fig 3.20). It is clear that at the sucrose concentrations present in most of these sweet beverages, the relative activity of the glucosyltransferases is very high indeed. In some cases, the sucrose concentrations would be sufficiently high to inhibit the enzyme, at least partly, if these concentrations were even reached in plaque (see 3.7.3 and 3.7.4).

3.7.3 Clearance of sucrose from saliva

The sucrose contents of sweet foods and confections can be readily determined by the sucrose assay technique described in section 2.3.3. However, what is more important in this study is the rate at which the sucrose is dissolved in saliva (for solid foods) and cleared from the mouth. This might reasonably be expected to vary, depending on the physical nature of the food in question.

In order to determine these rates, sucrose concentrations in the total oral fluid ('whole saliva') were determined, during and after consumption of various sucrose-containing sweet foods and drinks. The subject for these experiments was a normal healthy adult male accustomed to consuming moderately sweet foods and drinks. In order that the results might be as 'realistic' as possible, he was instructed to eat or drink the sample, ad libitum, in what he considered to be a 'normal' way. The samples were consumed and the saliva sample

was collected and centrifuged as described previously (section 2.3.8). Sucrose concentrations were then determined as described in section 2.3.3.

Figs 3.25 to 3.28 show the sucrose concentrations in whole saliva during, and for 10 min after, consumption of various foods and drinks. The graphs also show the relative enzyme activity, as a percentage of maximum velocity, interpolated from fig 3.20, which would occur in these concentrations of sucrose.

While 'Coca-Cola' was in the mouth (fig 3.25), the sucrose concentration in saliva was 330-350 mmol/l (cf. Table 3.8). At these concentrations, the glucosyltransferases would be about 53% active. After the drink was consumed, sucrose concentration dropped rapidly, reaching about 1 mmol/l after 3 min. During this clearance phase, glucosyltransferase activity would reach maximum for a short time but enzyme activity would have effectively ceased after about 10 min.

While a boiled sweet was sucked the salivary sucrose concentration was between 80 and 120 mmol/l (fig 3.26). This would give maximum glucosyltransferase activity. The concentration of sucrose varied little over this period. This is probably because the subject unconsciously regulated the rate of dissolution of the sweet in order to maintain the subjectively most 'pleasant' level of sweetness. In fact, many individuals, including those with a 'sweet tooth', find very high concentrations of sucrose unpleasant or even bitter. [The psychology of the pleasantness (hedonics) of sweet materials has been reviewed by Moskowitz (1974).] Sucrose was not completely cleared from the mouth until about 10 min after the sweet was finished.

As shown in fig 3.27, soft toffees are dissolved, due to

FIG 3.25 CLEARANCE OF DIETARY SUCROSE FROM SALIVA

"COCA COLA"

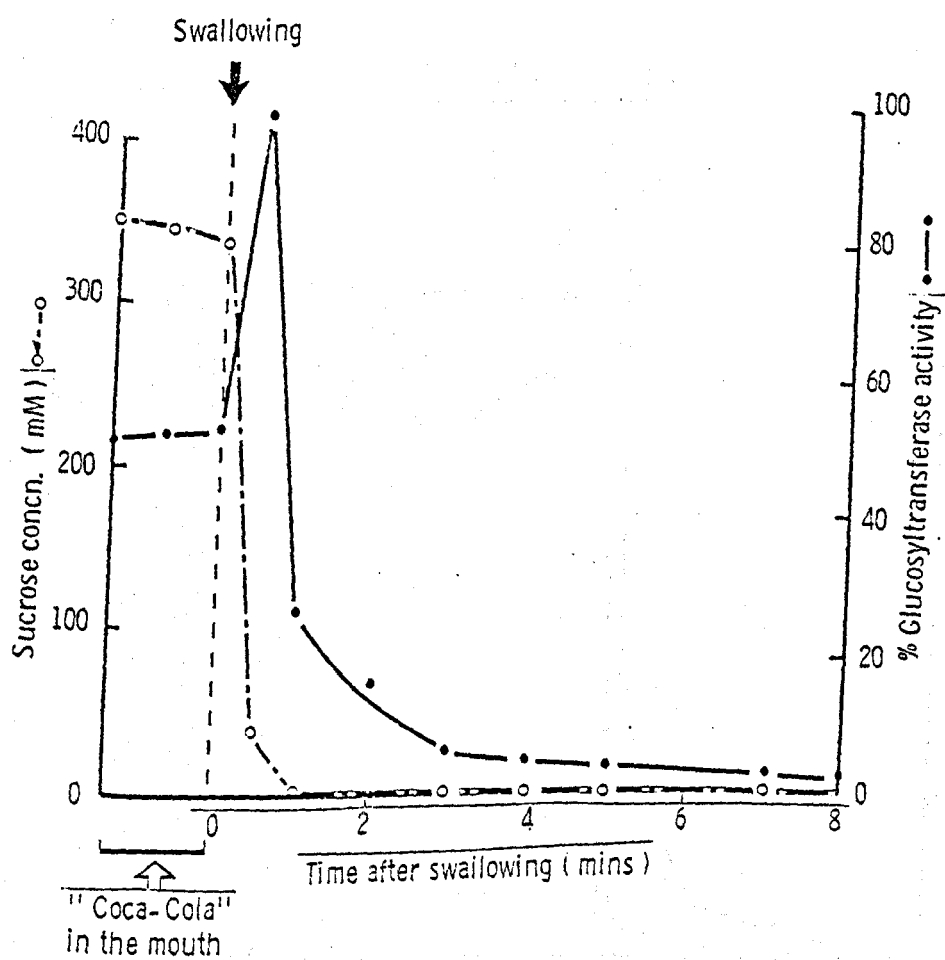


Fig. 3.26 CLEARANCE OF DIETARY SUCROSE FROM SALIVA

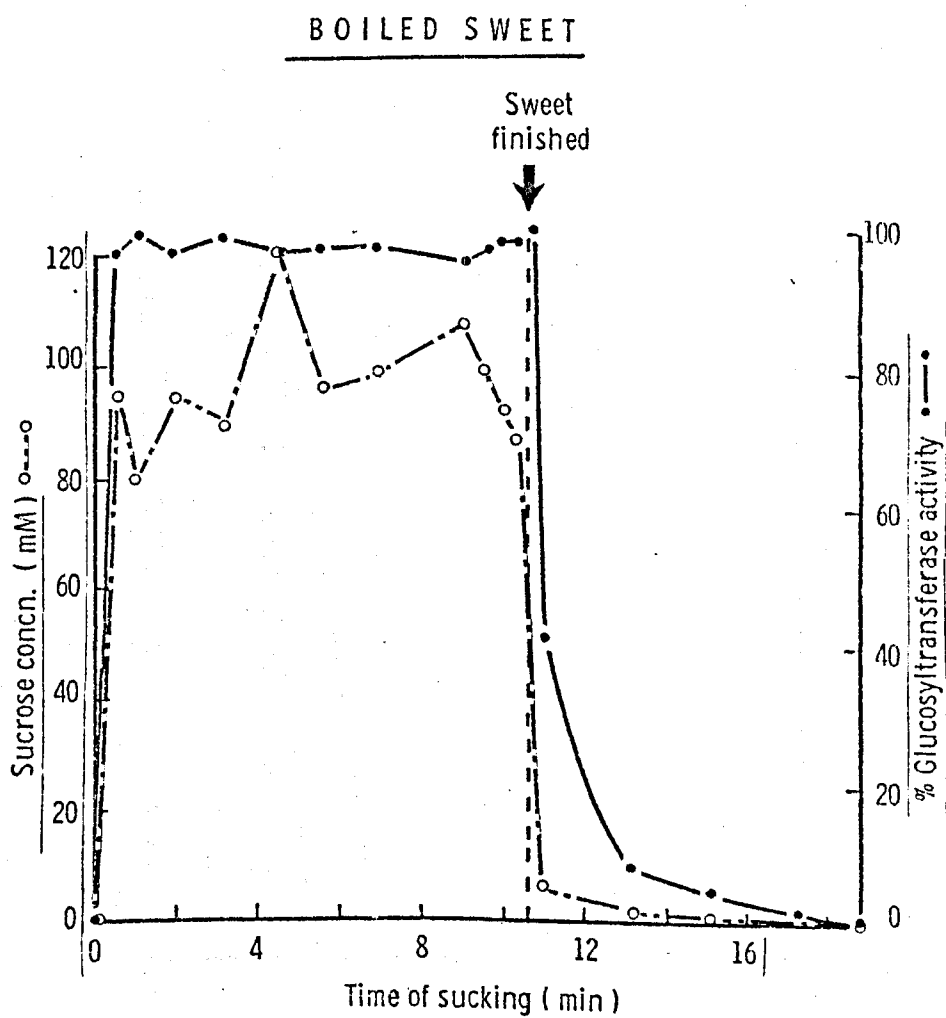
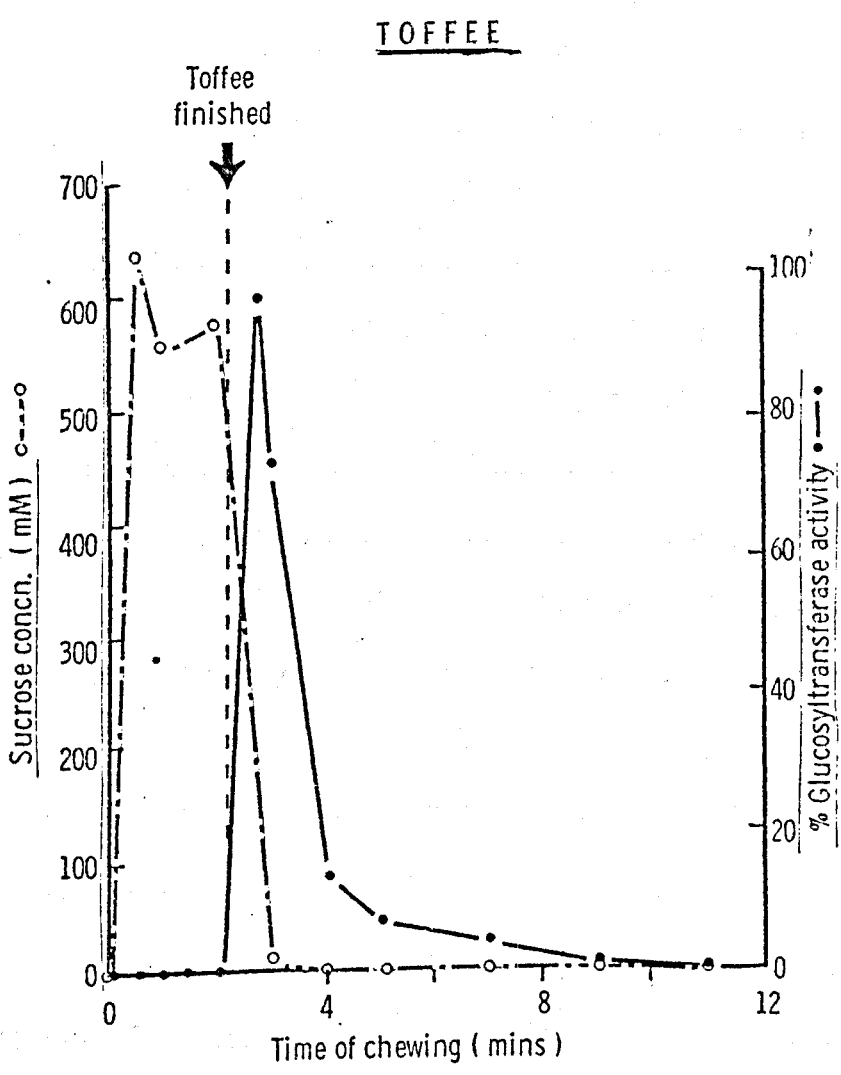


Fig. 3.27 CLEARANCE OF DIETARY SUCROSE FROM SALIVA



mastication, much more rapidly than boiled sweets. Thus, the maximum concentration of sucrose in the saliva (about 600 mmol/l) was much higher. Synthesis of high molecular weight glucan would be almost completely inhibited at this concentration. Sucrose was cleared after about 10 min and high glucosyltransferase activity would only occur during the clearance phase.

While chocolate was being chewed (fig 3.28), the salivary sucrose concentration varied greatly, slowly reaching a peak of 531 mmol/l after 2 min and decreasing slowly thereafter, even before the sweet was finished. This effect was reproducible. The reason for the decrease is not clear but it was not due to increased salivary flow since this was fairly constant (2.0-2.5 ml/min) throughout the experiment. Some of the chocolate may have been inadvertantly swallowed in between saliva collections since chocolate is more friable and more readily dispersed in saliva than toffee or boiled sweets. After the chocolate was finished, sucrose was cleared slowly, taking about 8 min to drop from 73 mmol/l to about 1 mmol/l. The reason for this is also not clear but is presumably due to the physical properties of the chocolate. As a result, the calculated glucosyltransferase activity decreases comparatively slowly.

While tea containing a (calculated) sucrose concentration of 117 mmol/l ('one spoonful per cup') was in the mouth, the concentration of sucrose in the saliva was 115-120 mmol/l and glucosyltransferase activity was almost maximal (see fig 3.29A). It is interesting to note that with more heavily sweetened tea, giving a salivary sucrose concentration of 353-357 mmol/l, glucosyltransferase activity would be only about 50% of maximum (see fig 3.29B); moreover, the sucrose was

Fig. 3.28 CLEARANCE OF DIETARY SUCROSE FROM SALIVA

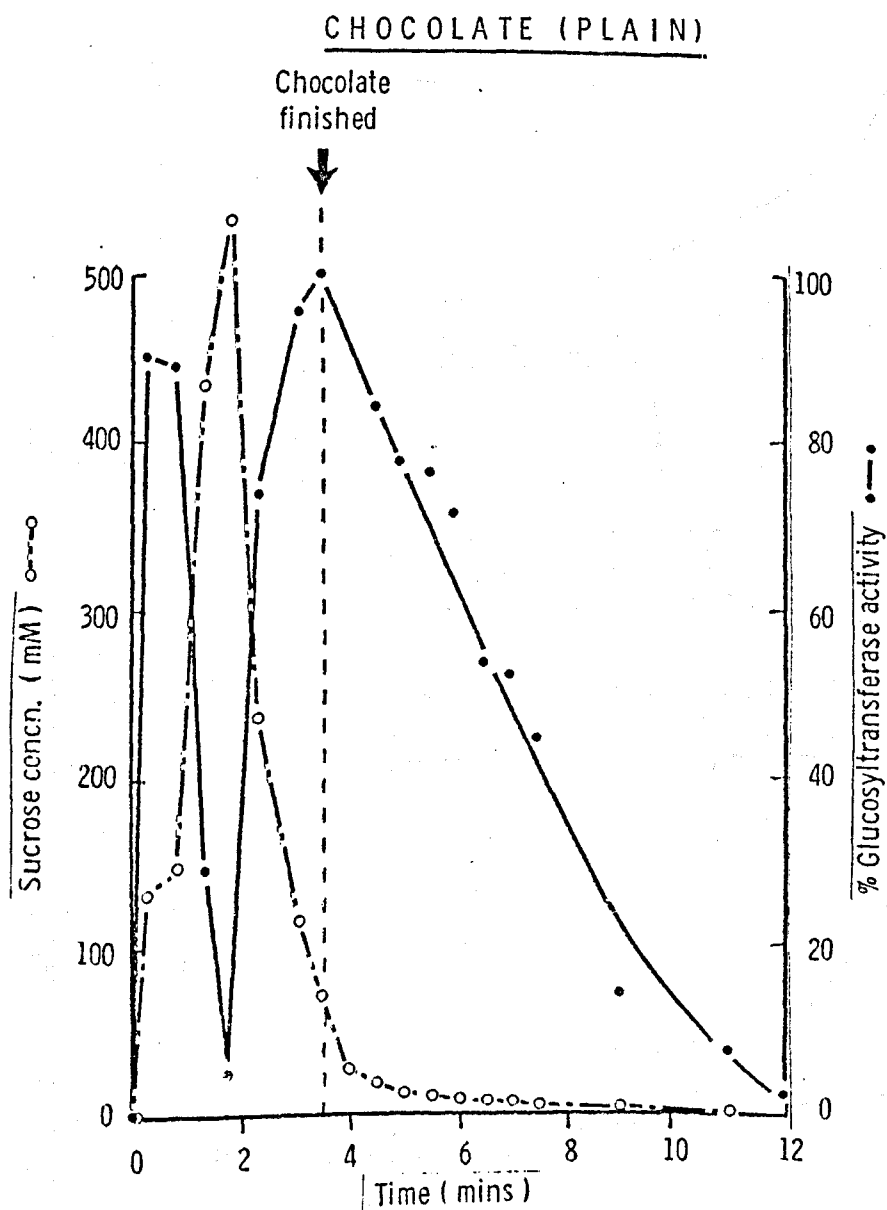
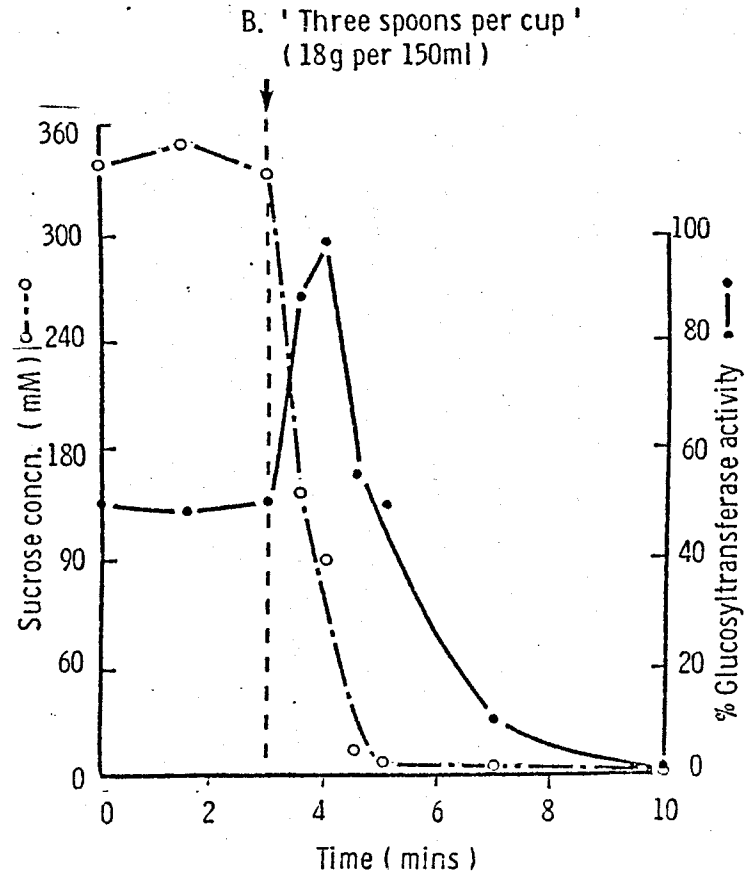
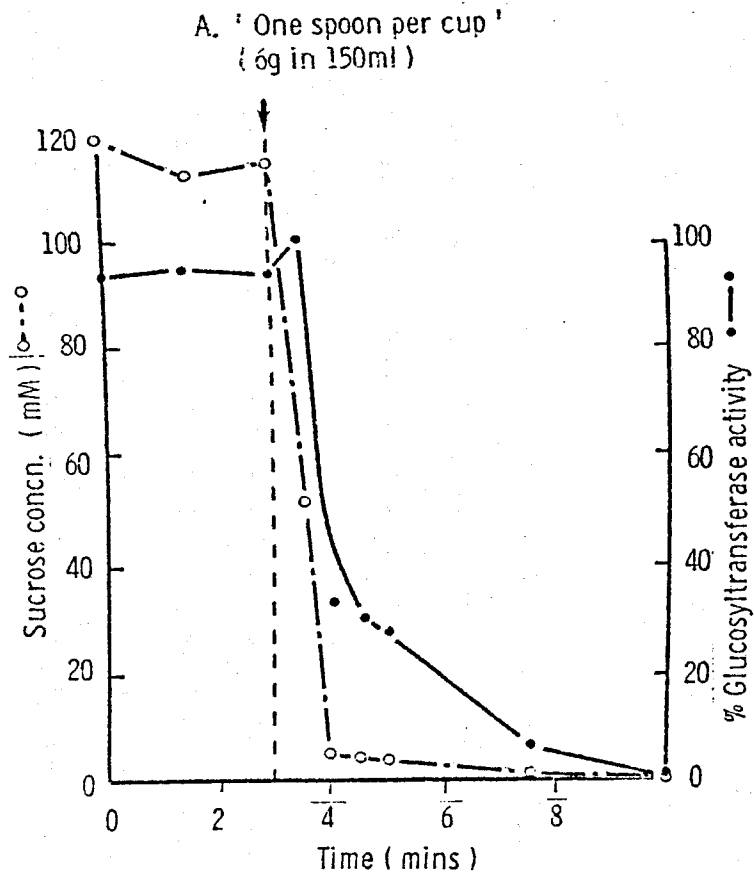


Fig. 3.29 CLEARANCE OF DIETARY SUCROSE FROM SALIVA -- TEA



cleared almost as rapidly as with 'one spoonful'. Thus, while the extra sucrose may be dietetically inadvisable, it is not necessarily any more cariogenic.

3.7.4 Clearance of sucrose from dental plaque

The patterns of clearance of sucrose from whole saliva can now be fairly easily determined (see 3.7.3). Studying these has provided useful measures of the oral sucrose concentrations produced with various sweet foods and drinks. However, the activities of the glucosyltransferases and other sucrose-metabolising enzymes found in dental plaque are only approximated by this method. They can only really be estimated accurately by measuring the sucrose concentrations which actually occur in plaque. This is technically much more difficult than the salivary studies described in section 3.7.3 because only very small amounts of plaque (typically, 20 mg) can be obtained from any one mouth.

In attempting to study clearance of sucrose from plaque, it did not seem advisable to use plaque pooled from several individuals. The amount, thickness and macroscopic texture of plaque vary from one individual to another. It was thus impossible to predict how much rates of clearance would vary between individuals. In all the experiments described here, the same subject, a healthy adult male, not receiving dental treatment, was used. The subject rinsed his mouth for 30 sec with 10 ml of a 20% w/v (584 mmol/l) sucrose solution in distilled water and then spat out the solution. On the basis of diffusion studies, 30 sec should be long enough for a typical plaque 100 μ m thick to be 90% saturated with the sucrose solution (S.G.McNee, personal communication). At various times after the rinse, plaque

was removed using a metal scraper and placed in a humid container in an ice bath. When all the plaque needed was collected, it was weighed and the extracellular fluid separated as described in section 2.3.9.

In preliminary experiments, about half of the whole mouth plaque was collected before the rinse (zero-time sample) and about half 5 min after the rinse. The contents of the centrifuge tubes were diluted to the volumes shown (see Table 3.9) and assayed for sucrose. As shown in table 3.9, all the extinctions obtained were below the limit of resolutions of the assay (ΔE of 0.03, equivalent to 15 nmol sucrose/assay). There are various possible explanations for this. As explained in the preceding paragraph, it is unlikely that the sucrose did not penetrate the plaque. It is also unlikely that the plaque had metabolised all the available sucrose, since, as shown in section 3.7.3, there would still be a fairly large reservoir of sucrose, even beyond 5 min. The most reasonable explanation of the observations is that the fluid which passed through the separating filter (see fig 2.9) did not reach the centrifuge tube; it was probably caught and dried on the underside of the filter holder. Indeed, no fluid was ever visible in the centrifuge tube, to the naked eye. Hence, the experimental procedure was modified.

In order to improve the yield of plaque ECF, the plaque samples were overlayed with 0.5-1.0 ml of 0.9% w/v (154 mmol/l) NaCl solution. When the assembly was centrifuged, the saline solution rinsed the plaque and carried any extracellular fluid through to the centrifuge tube.

The standard incubation volume for the sucrose assay is 3.02 ml. In order to improve the sensitivity, the assay volumes were scaled

Table 3.9

EXPERIMENTS ON SEPARATION OF SUCROSE FROM DENTAL PLAQUE

Time after sucrose rinse (min)	Diluted Volume of ECF (ml)	Experiment 1 Wt. of Plaque (mg)	$\Delta E_{\text{sucrose}}$	Diluted Volume of ECF (ml)	Experiment 2 Wt. of Plaque (mg)	$\Delta E_{\text{sucrose}}$
0	1.0	4.5	0.006	0.5	4.45	0.021
5	1.0	7.4	0.001	0.5	2.15	0.041

NOTES

- (i) $\Delta E_{\text{sucrose}} = \Delta E_{\text{total}} - \Delta E_{\text{glc}}$, at 340 nm, in the sucrose assay (see section 2.3.3).
- (ii) In both experiments, plaque ECF was separated by centrifugation and diluted to the volume shown. 0.1 ml of the diluted ECF was used as the sample in the sucrose assay (incubation volume = 3.02 ml).
- (iii) Limit of resolution of the sucrose assay corresponds to $E_{\text{sucrose}} = 0.030$.

down to semi-micro (1.71 ml) and micro (0.302 ml) levels. The various incubation mixtures were prepared as shown in Table 3.10.

The assay procedure was not operable at the micro level. With standard sucrose solutions, no NADPH was formed and this small assay volume appeared to be below the limits of reproducibility of the assay. However, the assay operated satisfactorily at the semi-micro level and this was used in all subsequent experiments on clearance of sucrose from dental plaque.

Table 3.11 shows the results of 4 such experiments. The subject rinsed his mouth with 20% w/v sucrose for 30 sec and whole mouth plaque was collected at various times after this. Column (1) shows when plaque collection was started, expressed as the time after the start of the sucrose rinse and column (2) shows when plaque collection stopped. Column (3) gives the difference between these, the total time required for collection of plaque. Obviously it is desirable to keep this time as short as possible and times were reduced with practice. Column (4) shows the concentrations of sucrose and glucose found, expressed as nmoles per mg wet weight of the whole plaque sample. Column (5) shows the same data, calculated as mmol/l in the plaque ECF. The calculations are based on the assumption that plaque ECF is 31.2% of the volume, as determined by Edgar and Tatevossian (1971), and that the specific gravity of the plaque is 1.04 (see section 3.8.2). This also assumes that all the glucose and sucrose measured were extracellular and that none was expressed from bacterial cells by the centrifugation.

There are certain difficulties in interpreting the results in Table 3.11. The accuracy of the values in column (5) is dependent upon the accuracy of the value for the proportion of extracellular

Table 3.10

COMPOSITION OF SUCROSE ASSAY INCUBATION MIXTURES

	VOLUME (ml)		
	MICRO	SEMI-MICRO	'NORMAL'
Sample	0.08	0.20	0.10
Invertase Solution	0.02	0.10	0.20
NADP, ATP Buffer Solution	0.1	0.5	1.0
Distilled H ₂ O	0.10-0.18 ⁽ⁱ⁾	0.7-0.9 ⁽ⁱ⁾	1.7-1.9 ⁽ⁱ⁾
Hexokinase/ Glucose-6- phosphate de- hydrogenase Solution	0.002	0.010	0.020
Total Volume	0.302	1.51	3.02

NOTES

- (i) Depending on the tube. For sucrose estimation, all the reagents are present. For the blank, the sample is omitted, and for glucose estimation, the invertase. H₂O is used to make up the difference in volume.

Table 3.11

CLEARANCE OF SUCROSE FROM DENTAL PLAQUE

(1) TIME AFTER START OF SUCROSE RINSE (min)	(2) TIME AT END OF COLLECTION (min)	(3) TIME FOR COLLECTION OF PLAQUE (min)	CONCENTRATIONS OF GLUCOSE AND SUCROSE			
			(4) nmol/mg wet wt of plaque Glucose	wt of plaque Sucrose	(5) mmol/l in ECF Glucose	Sucrose
0.75	3.20	1.45	12.9	90.2	43.1	301
1.0	3.42	2.42	3.3	12.8	10.6	67.9
1.0	3.92	2.92	8.4	26.3	28.1	88.3
1.0	4.25	3.25	8.2	5.87	27.4	19.6
5.25	7.50	2.25	4.75	1.90	15.8	6.4

NOTES

- (i) The values in column (3) are the differences between those in (1) and (2).
- (ii) Concentration of sucrose in rinse = 584 mmol/l (20%, w/v).
- (iii) If the plaque ECF is saturated with the 584 mmol/l sucrose solution, the sucrose concentration in the plaque will be 190 nmol/mg wet wt. of plaque assuming that the ECF is 31.2% of the volume of the plaque (Edgar & Tatevossian, 1971) and that the plaque density is $1.04 \times 10^3 \text{ kg.m}^{-3}$ (see table 3.12).
- (iv) Each time-point and plaque sample represent a different experiment.

fluid in plaque. Ideally, one should determine the proportion of plaque extracellular fluid for each subject examined but this is not feasible. Also, the time required to collect the plaque is a large proportion of the duration of the experiment. This makes it difficult to plot the results graphically. The clearance of sucrose from plaque is an exponential (or more probably, a multiple exponential) decay, therefore it is not satisfactory to plot the midpoint between the starting and finishing times of the plaque collection.

The rinsing time used (30 sec) is probably sufficient for the sucrose to penetrate most of the plaque. The thickness of plaque varies considerably, but plaque 100 μm thick would, by diffusion, be about 90% saturated with a sucrose solution (S.G. McNee, personal communication). This assumes that the effects of metabolism on penetration by sucrose are negligible. Given the comparatively large volume of rinse (10 ml) and the very high concentration of sucrose (584 mmol/l) used, this assumption is probably justified. The experimental findings confirm this. Shortly (15 sec) after the sucrose rinse, plaque was collected over a period of 1.45 min. The sucrose concentration in this sample was very high (90.2 nmol/mg plaque, or about 300 mmol/l in the ECF). The concentration dropped rapidly thereafter and so was probably even higher while the rinsing solution was in the mouth.

This was a preliminary study but it is possible to make some conclusions regarding penetration of plaque by sucrose. The findings of McNee et al. (1979), along with those reported here, show that this penetration and subsequent clearance of sucrose are not limited by diffusion. From the limited results obtained, it appears that clearance

of sucrose from plaque and saliva occur at roughly the same rates (cf. section 3.7.3, especially figs 3.25 and 3.29). Thus even if any clearance of sucrose due to bacterial metabolism is very high, the concentrations of sucrose in plaque will probably be maintained by the large reservoir of sucrose in saliva.

Finally, it would be wrong to place too much emphasis on the values shown in table 3.11. However, they do suggest that the concentrations of sucrose which occur in plaque can be sufficient for high glucosyltransferase activity.

3.8 Pycnometry

3.8.1 Introduction

It is difficult to measure the volume of plaque and plaque extracellular fluid directly. However, both of these substances can be conveniently weighed on a microbalance. If the densities are known, their volumes can then be calculated. These densities were therefore determined using the organic solvent density gradient technique described in section 2.3.6.

It is also difficult to measure volumes of various oral fluids, either because only tiny amounts are secreted (e.g. minor gland saliva), or because they are too viscous to be pipetted (e.g. sublingual saliva). The study was thus extended to include whole saliva and the technique has now been used to determine the density of saliva secreted by individual glands (Lindsay et al., 1978).

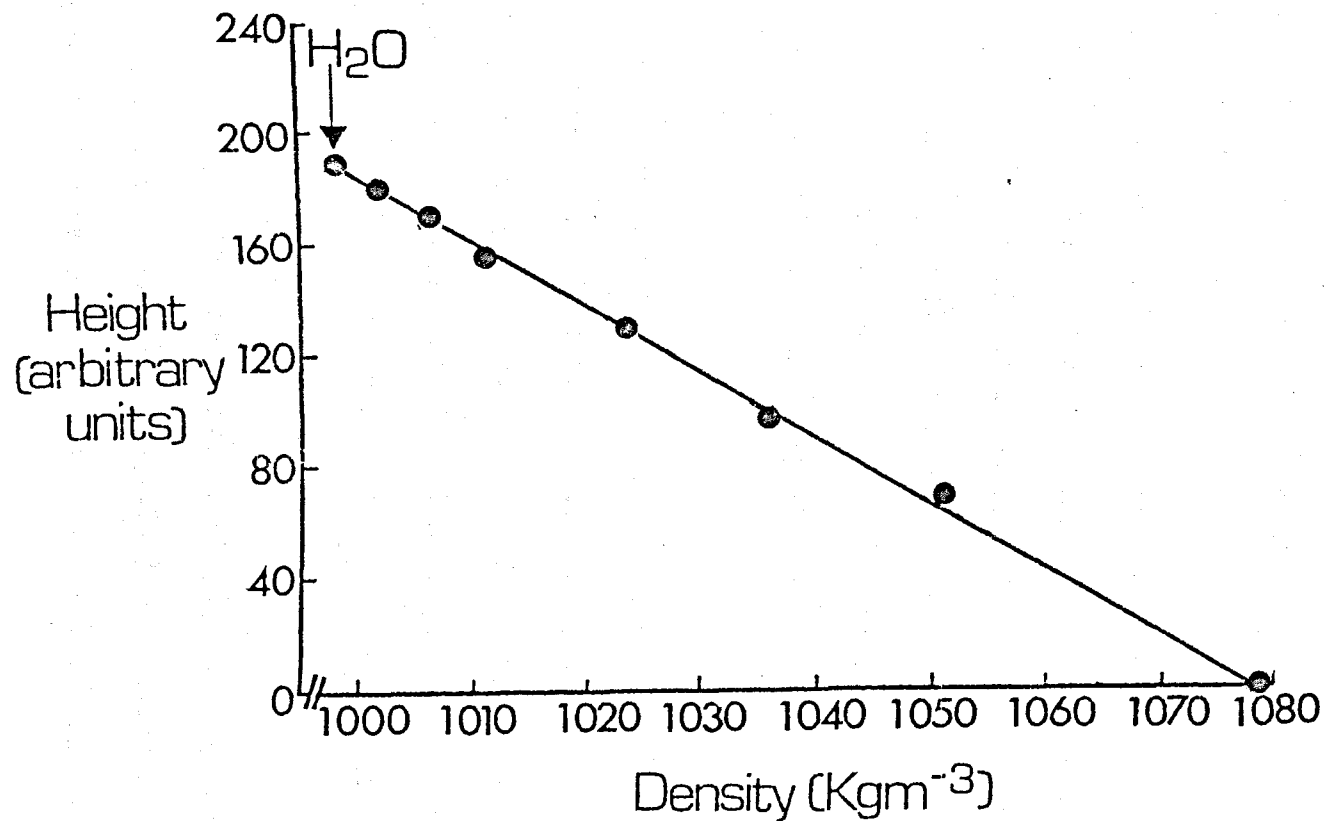
Density gradients were calibrated using standardised solutions of CuSO_4 . (Other solutions, such as sucrose, NaCl or KI can be used but this does not affect the results obtained.) Fig 3.30 shows the calibration curve obtained for one gradient.

In all cases, the samples applied to the column could be extremely small (even less than 1 μl) as long as they were visible to the naked eye. In fact, because the positions of drops are measured through their mid-points, it is easier to read the positions of very small drops of samples.

3.8.2 Densities of plaque and saliva

Dental plaque and whole (unstimulated) saliva were studied. Plaque was whole mouth plaque grown for 24 h or 3 h and collected from

Fig.3.30 CCl_4 /Petroleum Ether Density Gradient- CuSO_4 Standards



several subjects 2 h after food or drink by scraping with a nickel spatula. The solid (cells and other solid materials) and aqueous (extracellular fluid) phases of plaque were separated by centrifugation at 15,000 g at 4°C for 15 min in 1 ml glass centrifuge tubes. Tubes were sealed with cellophane to prevent condensation or evaporation. Plaque and separated plaque solids could be placed directly into solvent gradients; the samples did not break up and remained stable in the gradients for several days.

Whole saliva was collected in centrifuge tubes on wet ice and centrifuged at 15,000 g at 4°C for 30 min to remove debris. Plaque and saliva samples applied to gradients were allowed to equilibrate to room temperature (20°C) in the gradient before positions of the drops were noted. For the best possible accuracy, the positions of drops of density standards were read simultaneously with the position of samples.

Table 3.12 shows the results obtained. In each case where plaque solids were examined, the density was 1.01 times that of the corresponding whole plaque sample. The density of the fluid extracted from plaque, even under high speed centrifugation (15,000 g) may be taken as 1.00, which is sufficiently accurate for most practical purposes.

The specific gravity of whole saliva (1.0009) was slightly less than that published for parotid saliva (1.0033 ± 0.00115) which actually has less total dissolved solids than whole saliva (Mason & Chisholm, 1975).

Table 3.12 DENSITIES OF ORAL FLUIDS AND DENTAL PLAQUE

SAMPLE	MEASURED DENSITY (kg.m^{-3})	
	Mean \pm S.D. (n = number of samples)	
Dental plaque (24 h)	1039 \pm 10	(8)
Dental plaque (3 h)	1042	(3) Range 1040-1043
Plaque solids (24 h)	1045	(3) Range 1042-1047
Plaque extracellular fluids (24 h)	995	(2) Range 994-996
Whole (unstimulated) saliva	995.9 \pm 0.7	(9)

(i) The absolute density of water at 20°C is approx. 998.2 kg.m^{-3} . It was measured gravimetrically in this study and was found to be 995.0 \pm 0.2 kg.m^{-3} (S.D.; n = 12).

(ii) All densities were measured at 20°C.

3.9 ZrOCl_2 Assay for Fructose

The zirconyl chloride assay for fructose [see Section 2.3.3 (ix)] was investigated as an attempt to find a rapid and specific assay for fructose in measurement of glucosyltransferase activity.

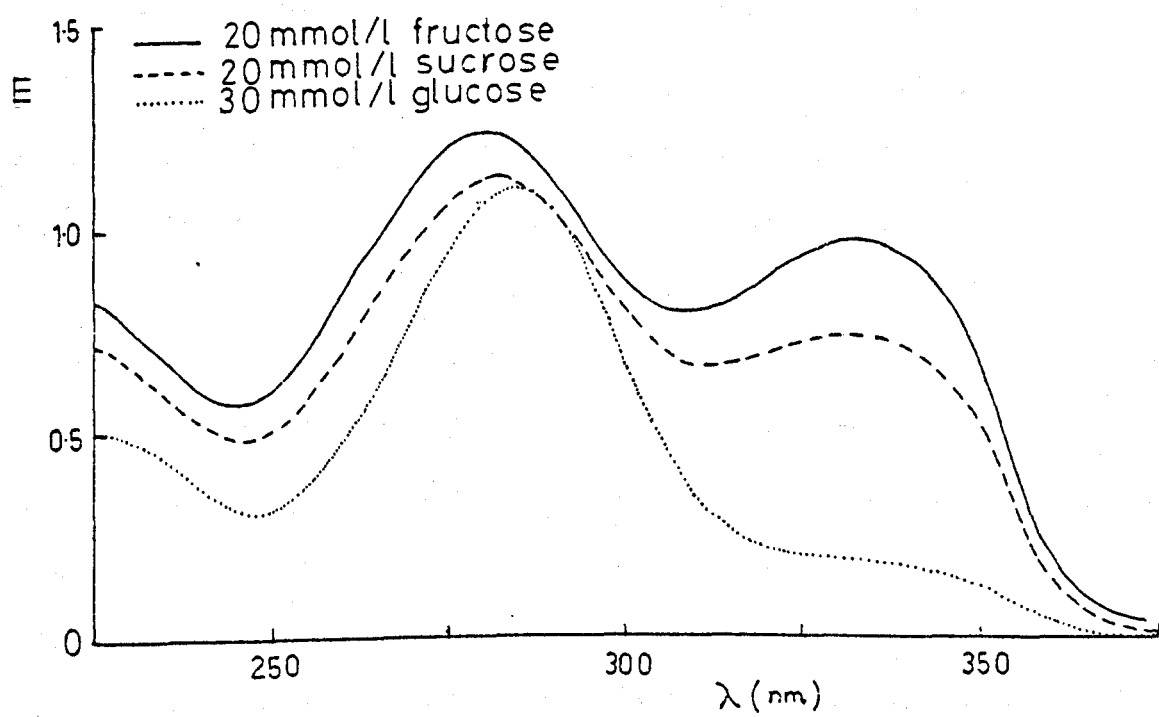
Samples of fructose, glucose and sucrose were incubated with the ZrOCl_2 reagent, as described in 2.3.3 (ix). The mixture was diluted to 3 ml with distilled water and UV spectra obtained.

As can be seen from fig 3.31, all three sugars reacted with the ZrOCl_2 reagent. The following were wavelengths which gave extinction maxima.

Fructose	281 nm,	332 nm
Sucrose	282 nm,	332 nm
Glucose	285 nm	

The extinction coefficient with glucose, at 332-334 nm, is much lower than that of fructose. In absence of invertases, the effect of glucose in estimations of glucosyltransferase activity would be negligible. However, sucrose gives a spectrum similar to that of fructose. When glucosyltransferase is incubated with sucrose in assays for enzyme activity, the sucrose concentration is of necessity in great excess of the concentrations of enzyme and product (fructose). Thus, any fructose released by glucosyltransferases from sucrose would not be detectable because the extinction coefficients with fructose and sucrose are similar at all wavelengths studied; the change in extinction due to release of fructose would be negligible. Hence, this technique would be useless for estimating glucosyltransferase activity.

Fig 3.31 UV ABSORPTION SPECTRUM IN ZrOCl_2 ASSAY



4. DISCUSSION AND CONCLUSIONS

S. sanguis produces adhesive extracellular glucans from sucrose. As discussed earlier (Section 1.2.3 - 1.2.5), these glucans are important in formation of plaque because they allow other species of bacteria (notably S. mutans) to bind to the surface of growing plaque. Thus, in order to understand the mechanism of cariogenesis (and hopefully to inhibit the process), it will be useful to characterise the glucosyltransferases, the enzymes which synthesise these glucans.

At the beginning of the project, the finding of Beeley and Black (1977), that glucosyltransferase production by S. sanguis 804, in batch culture, is triphasic, was confirmed. The extracellular glucosyltransferases of plaque streptococci are considered to be constitutive because they are produced in the absence of sucrose. However, the triphasic pattern demonstrates that the synthesis, or secretion, of these enzymes is regulated by more subtle mechanisms. Beeley and Black (1977) showed that enzyme preparations from each phase produced glucans of differing structures. The mechanisms whereby such different enzyme activities are produced at different ages of culture are unknown. However, it is clear that caution must be exercised in studying the glucans of S. sanguis. Varying conditions, such as age of culture, availability of carbon source and pH, may greatly affect the results obtained, whether in batch culture, continuous culture, or even in plaque itself.

The original aim of this project was to isolate and characterise the glucosyltransferases of S. sanguis 804. Phase II of glucosyltransferase production, which occurs at the end of logarithmic growth phase, when the medium was depleted of glucose, was investigated. Many workers have used the same basic techniques (i.e. ammonium sulphate precipitation

followed by hydroxylapatite chromatography) to purify the glucosyltransferases of S. sanguis and S. mutans. However, most have reported low recoveries of enzyme activity after these and/or other procedures. Typical recoveries were between 10% (Long, 1971) and 15% (Carlsson et al., 1969) although Kuramitsu (1975) reported 32% recovery of activity (from S. mutans GS-5) using ammonium sulphate followed by either gel filtration (on Bio-Gel A-15) or hydroxylapatite chromatography. The considerable purification reported by some workers (e.g. 1500-fold with 13% recovery, by Chludzinski et al., 1974) might have been much enhanced by better recoveries. Such poor recoveries may be acceptable when dealing with a homogeneous (i.e. single-enzyme) system. However, the glucosyltransferases of S. sanguis and S. mutans are not homogeneous and are either multi-enzyme systems or contain several post-translationally modified forms of one enzyme (see 1.3.4). As a result, part of the reason for the low recoveries might be loss of one or more individual forms of glucosyltransferase during purification. In support of this suggestion, it has been shown that the molecular structures of the streptococcal glucans vary according to the degree of purification of the enzyme preparations used to synthesise them (Nisizawa et al., 1977; Ceska et al., 1972). This may be due to the loss of particular enzyme activities during purification. If this is the case, then the synthesis and properties of glucans by purified enzyme may differ drastically from those of glucans synthesised in vivo (i.e. in plaque) as well as those synthesised by crude preparations of enzyme, such as culture fluid.

Thus, the conclusions which one can draw from studies on highly purified glucosyltransferases may be very limited depending upon the recovery of the enzyme after purification. Even if recovery is 100%, the structure of the glucans may be affected by the conditions used to

synthesise them. For example, recent work by Mohan et al. (1979) suggests that the dextransucrase and mutansucrase activities of S. mutans may be due to single enzyme. The purified dextransucrase synthesised a water-soluble glucan ('dextran') but could be made to synthesise a water-insoluble glucan ('mutan') by raising the salt (ammonium sulphate) concentration.

In this study, the recovery of enzyme activity after ammonium sulphate precipitation varied between 67% and 100%. Some recoveries were apparently greater than 100%. This may have been because (unidentified) inhibitors were removed by the purification or because the purified enzymes synthesised more easily precipitable (or less water-soluble) glucans. The recovery of enzyme after hydroxylapatite chromatography was about 30% of that applied to the column; overall yield was thus only about 24%. However, Germaine et al. (1974) showed that trace acceptors appeared to be removed from preparations of S. mutans glucosyltransferases by hydroxylapatite chromatography. It was necessary to add acceptors (e.g. dextran) to achieve the maximum rate of synthesis of precipitable glucan. In the absence of dextran, sucrose would act as a glucosyl acceptor and eluted enzyme activity could still clearly be detected by measuring release of fructose, as in this study (see fig. 3.5). However, sucrose is an inefficient glucosyl acceptor (Chludzinski et al., 1976). Hence the rate of glucosyl transfer would be less than the maximum, which would occur with, say, saturating concentrations of dextran. Apparent recovery would thus be somewhat reduced.

It seems likely, then, that the procedures described here could adequately form part of a purification scheme for isolating the component enzymes of the glucosyltransferase system, without losing any

of the enzymes. However, I would suggest that it may be useful to incorporate, routinely, a good glucosyl acceptor compound (e.g. a soluble dextran) into the incubation mixture when assaying enzyme activity. With this modification, studies on both crude and purified enzyme preparations would not be complicated by uncontrolled variations in the concentration of trace acceptors. The stimulating effect of the acceptor would increase the sensitivity of assay. Dextran as acceptor might also be a useful 'carrier' for ethanol precipitations when measuring glucan production although in high concentrations it would give a high background if precipitated glucan were estimated chemically.

Hydroxylapatite chromatography (fig. 3.5) showed that S. sanguis 804 produces at least 3 distinct glucosyltransferases each with different specific activities. The differences in specific activity may be partly due to differences in specificity and affinity for substrate and in type of reaction catalysed. If the enzymes are composed of different polypeptides, then the properties of the enzymes (e.g. specific activity, K_m and V_{max}) would be expected to vary. On the other hand, the enzymes may also be the same gene products modified, for example, in bound carbohydrate chains, if they are glycoproteins. Whatever the explanation, if the enzymes synthesise different products, then the differences in specific activity are probably also caused by differences in acceptor requirements. For example, in the absence of other glucosyl acceptors, sucrose acts as an acceptor for 'dextransucrase-like' enzymes (Ebert & Schenk, 1968b; Chludzinski et al., 1976). Mutansucrases may not be able to use sucrose as an acceptor so readily. Any branching enzymes probably have a strict requirement for a glucan, or, at least,

a long chain oligosaccharide as acceptor. The findings of Walker (1972) suggest that α -1,3 branch linkages cannot be added to sucrose or trisaccharide acceptors. The shoulders on the peaks of enzyme activity eluted from hydroxylapatite may (if genuine) be attributed to unresolved glucosyltransferases. However, they may also represent a 'synergic' effect whereby enzyme, from one of two overlapping peaks, synthesises a 'primer' or other substance which stimulates the activity of enzyme from the other peak. For example, dextransucrases would synthesise the iso-maltodextrin chains necessary for action of branching enzymes.

I have already suggested that some suitable glucosyl acceptor, such as dextran, be incorporated into incubation mixtures for the assay of enzyme activity, in order that recoveries of enzyme activity after hydroxylapatite chromatography might be determined more accurately. This modification would also increase the sensitivity of the assay and, perhaps, reveal previously undetected minor peaks of enzyme activity (especially those with strict acceptor requirements) which might otherwise be lost in purification. Any false 'shoulders', due to the synergic effect described above, would disappear since no enzyme should require synthesis of a primer by another.

The extracellular glucosyltransferase systems of S. mutans and S. sanguis have been shown to contain several distinguishable enzymes (Guggenheim & Newbrun, 1969; Newbrun, 1971). This finding has been confirmed, in this study, by hydroxylapatite chromatography. The broad range over which the enzymes are precipitated by ammonium sulphate was consistent with this. So too was the finding that some glucosyltransferase activity appeared to bind 'irreversibly' to Sephadex while some did not bind at all. (In order to confirm this

reliably, it would of course be necessary to demonstrate that the Sephadex contained enzyme activity, rather than merely calculating the amount of enzyme 'lost' on the Sephadex column from the apparent recovery of eluted enzyme activity.)

Kuramitsu (1975) and Schachtele et al. (1977) isolated the various glucosyltransferases of S. mutans and were able to identify different enzymes which synthesised water-insoluble (α -1,3-linked) or water-soluble (α -1,6-linked) glucans. However they did not determine whether these enzymes differed in amino acid sequence or were merely modified forms of one protein.

Many workers believe that the glucosyltransferase system is composed of one enzyme. This belief is based either on the automatic assumption that there is a single 'dextransucrase' or on experimental evidence showing a single band of protein or enzyme activity in chromatography, isoelectric focusing or electrophoresis (e.g. Fukui et al., 1974; Long, 1971). This appears to conflict with studies which show a multiplicity of enzymes. One possible explanation, for which there is little direct evidence, is that the various glucosyltransferases form a multi-enzyme complex; in some purifications, such a complex might appear as a single protein species. It is also possible, as described above, that various observed enzymes are all one protein. This may have different conformations produced by changes in salt concentration (Mohan et al. 1979) or may have several specific chemical modifications. Feary and Mayo (1978) found that a one-step mutation abolished the glucosyltransferase activity of S. sanguis and concluded that the glucosyltransferase system was a single protein. However, this is not conclusive, because this may have been a regulatory mutation or a deletion mutation, either of which could affect several genes.

There is some limited evidence to suggest that the glucosyltransferase, fructosyltransferase and invertase activities of S. mutans form a multi-enzyme complex. The three activities appeared as a single peak by isoelectric focusing (Aksnes, 1977) and by gel-filtration (Bio-Gel P-300) and ion-exchange chromatography (Scales et al., 1975). Scales et al. (1975) called this a 'glycosyltransferase complex' and showed, by gel-filtration, that its molecular weight was 800,000, 41% of which was carbohydrate. This large molecular weight suggests an aggregate or complex of enzymes, rather than a single protein molecule.

In this study, the glucosyltransferases of S. sanguis 804 were separated as at least three peaks of enzyme activity, by hydroxylapatite chromatography. It will be useful to study the characteristics of these individual enzymes, such as K_m , pI and molecular weight. There are no obvious problems in studying any of these except molecular weight. The molecular weights cannot be determined by gel-filtration because the glucosyltransferases of S. sanguis 804 bind strongly to Sephadex, Sepharose and Bio-Gel (Carlsson et al., 1969). Similarly, sucrose density gradients could not be used for sedimentation velocity analysis because sucrose binds strongly to the enzymes, and the glucosyl moiety of sucrose is polymerised by them; this would probably lead to anomalous results. Other density gradient media were considered, but most are probably unsuitable, as discussed already (see 3.2.5). Metrizamide was found to inhibit the glucosyltransferases and to interfere with some of the assay techniques used in this study (see 3.2.5) and so it too is unsuitable.

One density gradient medium which is unlikely to have a deleterious effect on the glucosyltransferases is D_2O (deuterium oxide).

Gradients of D_2O and H_2O can be prepared and Trinick and Rowe (1973) have used such gradients to separate the filaments of vertebrate skeletal muscles by ultracentrifugation. There are various methods available to measure the shape of such gradients, such as refractometry (Rilbe & Peterson, 1968) or pycnometry (as described in 2.3.6).

Solutions in D_2O have an apparent pH of about 0.4 pH units less than the same solutions in H_2O and this property has also been used to measure the shape of D_2O gradients (Glascoe & Long, 1960; Fredriksson, 1975). However, although the high molecular weight muscle filaments could be separated on D_2O gradients, it remains to be seen whether or not the technique could be used for separation of smaller, globular proteins, such as the glucosyltransferases.

Although the individual glucosyltransferases produced by S. sanguis 804 were not investigated in this study, the partially purified (ammonium sulphate) preparations have yielded useful information about the glucosyltransferase system.

Many other workers have shown that the activities of the glucosyltransferases of S. sanguis and S. mutans are stimulated by added dextran (see 1.3.4). The same was found in this study (see 3.4.1). However, crude and partially purified preparations were all stimulated to about the same degree (2-4 fold) by T2000 dextran at what are probably saturating concentrations (see fig. 3.17). This agrees with the findings of Germaine et al. (1974). They showed that glucosyltransferase activity in cell-free culture fluid was stimulated 2-3 fold by dextran of molecular weight 10,000 at a concentration of 17 $\mu\text{mol/l}$. (K_m for the dextran was about 3 $\mu\text{mol/l}$). After ammonium sulphate purification, activity was stimulated only about 1-5 fold. Thus, with their preparations and with those used in this study, the degree of

stimulation was small and did not increase with purification. Enzyme activity was thus not dependent on acceptors and so the ammonium sulphate preparations probably still contained substantial amounts of glucosyl acceptor compounds.

It was, however, surprising to find that nigerose did not stimulate glucosyltransferase activity (see 3.4.2). One would expect nigerose and nigerodextrins to be efficient glucosyl acceptors for mutansucrases. There are various possible explanations. S. sanguis may not produce a mutansucrase activity, or only very small amounts of it. This remains to be proved but the extracellular glucans of S. sanguis do, in general, seem to resemble dextrans rather than mutans (Ceska et al., 1972; Beeley & Black, 1977). It is also possible (although unlikely) that any glucosyltransferases present which can use nigerose as an acceptor were already saturated with acceptors present in the preparations. Sucrose itself may be a more efficient acceptor for mutansucrase than nigerose is and, indeed, these enzymes may not be able to use nigerose at all. If nigerose is a glucosyl acceptor, then it may have a similar effect to maltose. Robyt and Walseth (1976) showed that, while maltose was an efficient acceptor, the products formed could not be elongated into glucan chains. This would explain why maltose inhibits glucan synthesis (Sharma et al., 1974; Newbrun et al., 1975). If the same mechanism holds for nigerose, then this would explain the slight inhibition of glucan synthesis by nigerose seen in fig. 3.18.

The effect of sucrose concentration on glucosyltransferase activity is perhaps the most important single property of these enzymes, both biochemically and clinically. It has been shown, in this study,

that the observed effects of sucrose concentration depend on the method used to determine enzyme activity. If enzyme activity is measured as release of fructose, all glucosyl transfer will be detected, no matter what the product. On the other hand, if activity is measured as production of precipitable polysaccharide, the assay technique will detect only those products which have a sufficiently high molecular weight for them to be precipitated by ethanol. Moreover, even with high molecular weight glucan, there are bound to be some losses of material during precipitation and subsequent washing; this will reduce the efficiency of detection of the glucans. It was not surprising, therefore, that the two assays gave different values of K_m for sucrose. The apparent K_m was lower when measured by fructose release (6 mmol/l) than when measured by production of precipitable glucan (about 20 mmol/l).

In formation of dental plaque, it is the production of high molecular weight glucans which is important. These are the molecules which are important in adhesion of bacteria in dental plaque (see section 1.2.4). As explained previously (see 1.3.4) increasing concentrations of glucosyl acceptors probably have little effect on K_m as measured by fructose but will decrease the K_m for glucan synthesis. Since dental plaque contains large amounts of glucans (and other possible acceptors), the value of the K_m for glucan synthesis may well be much smaller, in vivo, than that reported here. At saturating concentrations of acceptors, the K_m may approach that for release of fructose. Glucan synthesis by S. sanguis is important in early plaque formation. One can consider the K_m for this process to be between 6 and 20 mmol/l (i.e. between those measured by fructose release and glucan synthesis) and compare these values with concentrations of sucrose which are found in the mouth, and, in particular, in dental plaque.

Physiological concentrations of sucrose can affect glucosyl-transferase activity in another, more striking way. Glucan synthesis was strongly inhibited by high concentrations of sucrose (above 70-100 mmol/l) (see 3.5.2). Thus, very high concentrations of sucrose in the mouth may actually inhibit plaque formation. The extent of this effect will depend on patterns of diet and this is discussed below (see p. 125 et seq.).

The dextranases of L. mesenteroides are also inhibited by high sucrose concentrations (Hehre, 1946; Stringer & Tsuchiya, 1958; Ebert & Patat, 1962). Chludzinski et al. (1976) found that at high concentrations of sucrose, the activity of S. mutans was less than expected by conventional Michaelis-Menten kinetics, although there was no actual decrease in activity with increasing sucrose concentration; this effect was eliminated by addition of dextran. The inhibition is believed to be due to binding of sucrose molecules to a second site, the acceptor site, on the enzyme molecule (Ebert & Patat, 1962; Chludzinski et al., 1976). With sucrose as a glucosyl acceptor, there will be an apparent decrease in the rate of glucan synthesis because oligosaccharides will be the immediate products. The proposed mechanism of inhibition by sucrose is confirmed by the finding that very high concentrations of an acceptor compound (α -methyl glucoside) could eliminate the inhibition of the dextranase of L. mesenteroides by high sucrose concentrations (Stringer & Tsuchiya, 1958). The same mechanism probably also applies to the glucosyltransferases of S. sanguis 804 because, although glucan synthesis is inhibited, release of fructose is not affected. This suggests that the reaction products contain a higher proportion of oligosaccharide when synthesised in high concentrations of sucrose. It has been suggested that the

inhibition of invertase by high concentrations of sucrose is due to reduction in the concentration of water (Nelson & Schubert, 1928). The reduction of molecular velocities due to the high viscosity of strong sucrose solutions may also affect rates of reaction. These would probably affect all enzyme activity, not merely synthesis of high molecular weight glucan, therefore they may not contribute greatly to the inhibition.

As already described, substrate inhibition of L. mesenteroides dextranucrase may be lessened by adding acceptor although high concentrations of the acceptor were needed to eliminate it completely (Stringer & Tsuchiya, 1958). The glucosyltransferases of S. mutans were only slightly inhibited; velocity did not actually decrease with increasing sucrose concentrations (Chludzinski et al., 1978). This suggests that the enzyme preparations may still have contained some glucosyl acceptors. There are probably large amounts of glucosyl acceptors present in plaque. Therefore, it is difficult to predict the extent, in plaque, of any substrate inhibition of the S. sanguis glucosyltransferases.

The extent to which substrate inhibition occurs in plaque could be estimated in two ways. The rates of synthesis of extracellular glucan in plaque could be measured directly or one could compare the K_m for various acceptors (K_a) with that for sucrose (at the acceptor site rather than the donor site). The former approach presents practical difficulties. The isolation of the plaque glucans and variations between plaque samples would lead to large variations in experimental results. Metabolism of sucrose by other enzymes (e.g. invertase and fructosyltransferase) would complicate the kinetics of glucan synthesis. The second approach, comparison of K_a values, might help to show how

Fig. 4.1 DIXON PLOT FOR 'CLASSICAL' SUBSTRATE INHIBITION

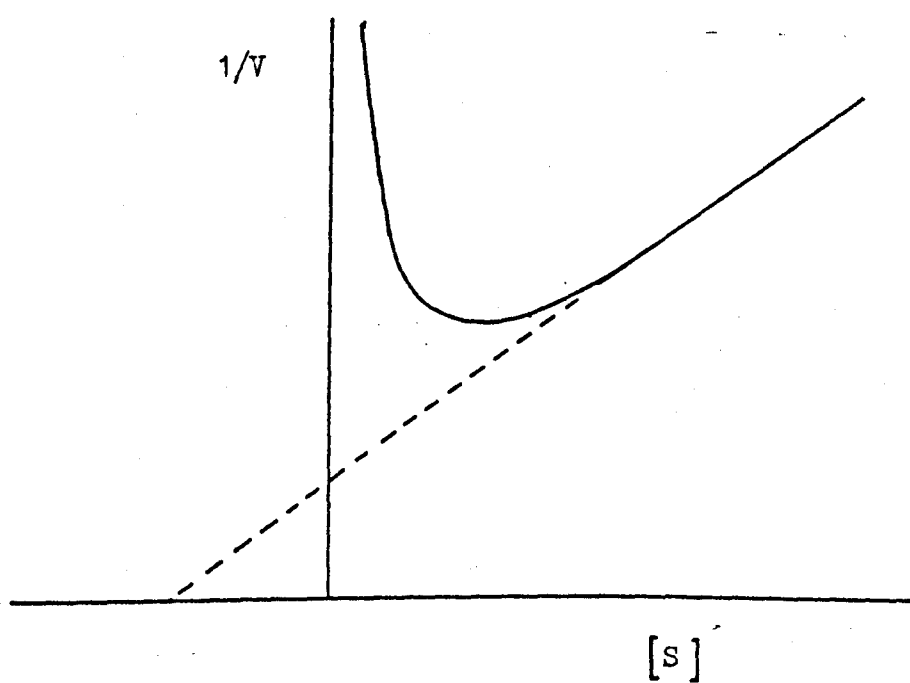
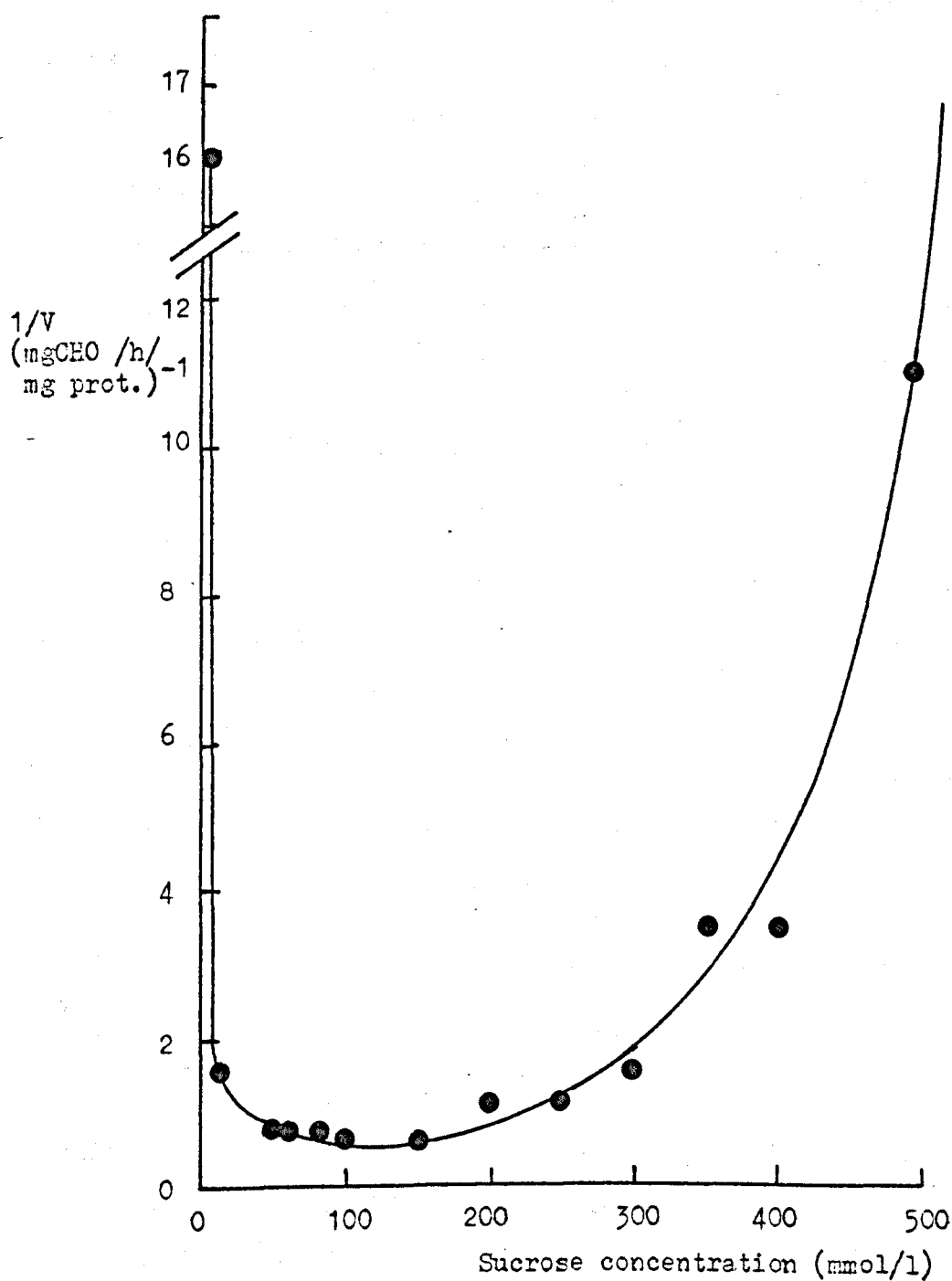


Fig. 4.2 DIXON PLOT FOR GLUCOSYLTRANSFERASES OF S. sanguis 804



NOTES

- (i) pH= 7.0
- (ii) Amount of protein/ assay= 55 μ g. Assay incubation volume= 1.0 ml.
- (iii) Enzyme used was ammonium sulphate purified preparation.

pattern. Enzyme velocity falls much more rapidly with increasing sucrose concentration than is usual. It is also possible to consider the curves in plots of V against $[S]$ as resulting from two component curves, one following Michaelis-Menten kinetics, and the other a pure inhibition curve, as shown in fig. 4.3. These curves can usually be extracted from sets of experimental data by computer line-fitting programmes, and K_i can then be calculated. However, the data obtained in this study could not be fitted to a single curve (Dr. I.P. Nimmo, personal communication). The *S. sanguis* system seems ~~to~~ more complex than others have been shown to be.

The most obvious complication with the glucosyltransferases is that ES_2 is a productive complex which can still lead to (oligosaccharide) products, albeit at a slower rate than with other acceptors. Stringer and Tsuchiya (1958) claimed that the rate equation described above was applicable to the dextranucrases of *L. mesenteroides*. However, the reliability of this conclusion is limited by the large variations in their experimentally determined values of K_i .

Ebert and Schenk (1968b) allowed for the fact that the ES_2 complex (with dextranucrase and sucrose) is productive. They found that the *L. mesenteroides* system was best described by the following equation;

$$V = \frac{V_{\max} \cdot [S]}{K_m + [S]} \cdot \frac{K_i}{K_i + [S]}$$

according to the following reaction scheme,

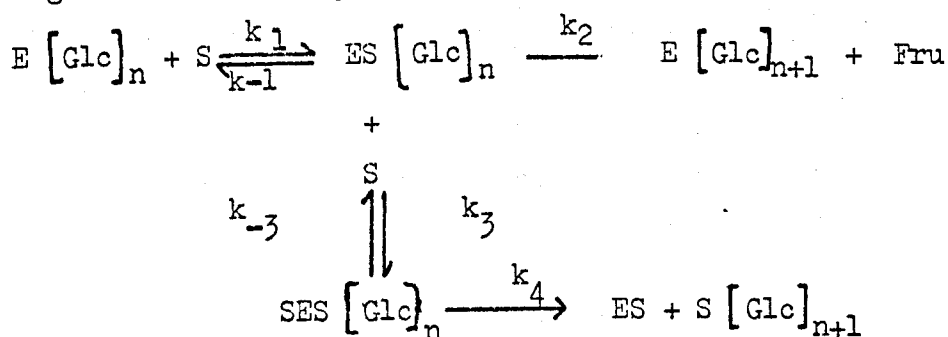
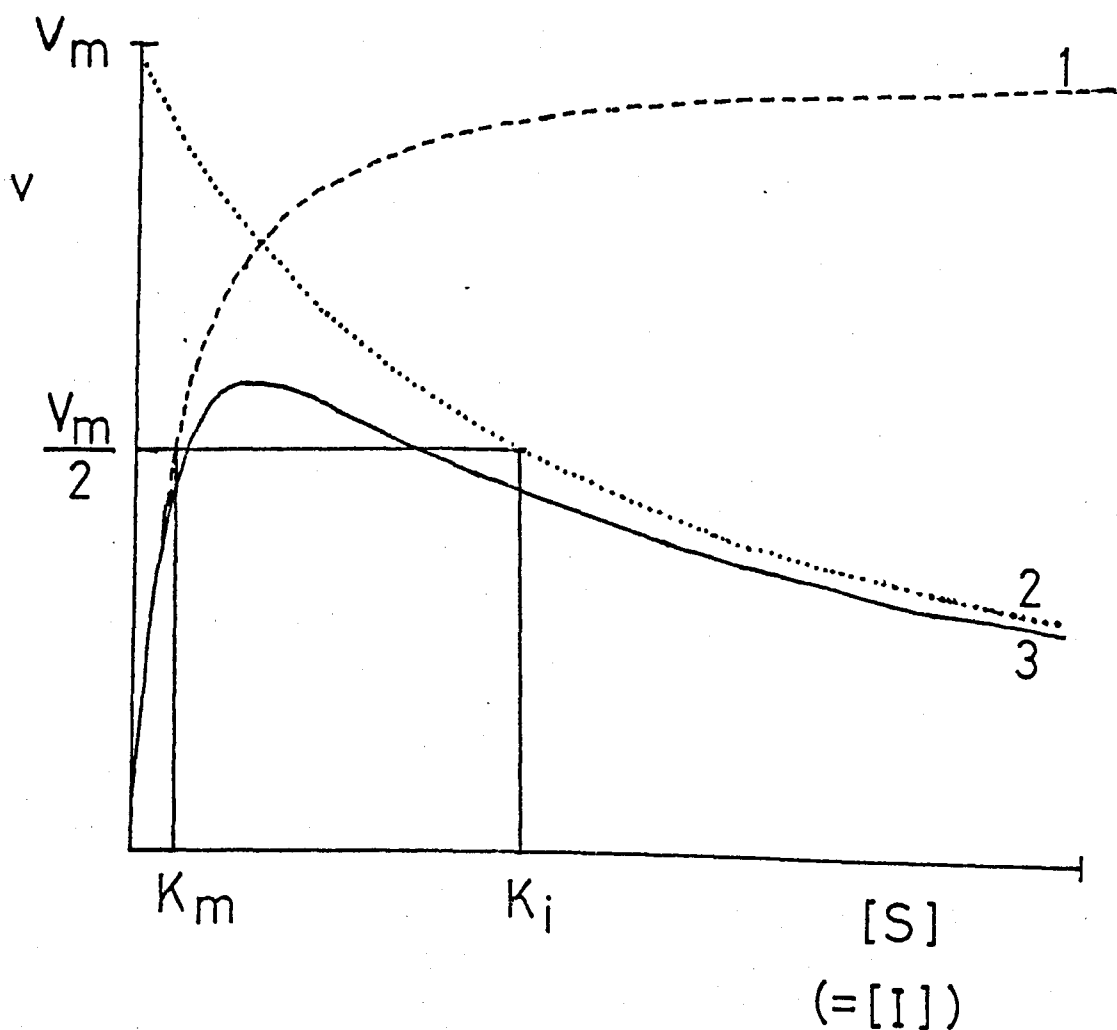


Fig. 4.3 SUBSTRATE INHIBITION



NOTES

Plot 1 represents normal Michaelis-Menten kinetics. Plot 2 describes uncompetitive inhibition; in cases of substrate inhibition, the inhibiting substrate molecule binds to the ES complex, therefore this form of inhibition is in some ways analogous to uncompetitive inhibition (Cornish-Bowden, 1976, p.66). Plot 3 represents substrate inhibition according to the rate equation of Ebert and Schenk (1968a), where $K_i = 10K_m$.

Converted to the form used by Cornish-Bowden (1976), the rate equation becomes:

$$V = \frac{V_{\max} [S]}{K_m + [S] + [S]^2/K_i + K_m \cdot [S]/K_i}$$

It should be noted that there is little evidence to support the idea of an SES[Glc]_{n+1} complex. It seems more likely that the second substrate molecule would bind to the acceptor site in place of the acceptor glucan chain, at high sucrose concentrations. However, the rate equation derived by Ebert and Schenk describes the experimental results for L. mesenteroides dextranucrase fairly well.

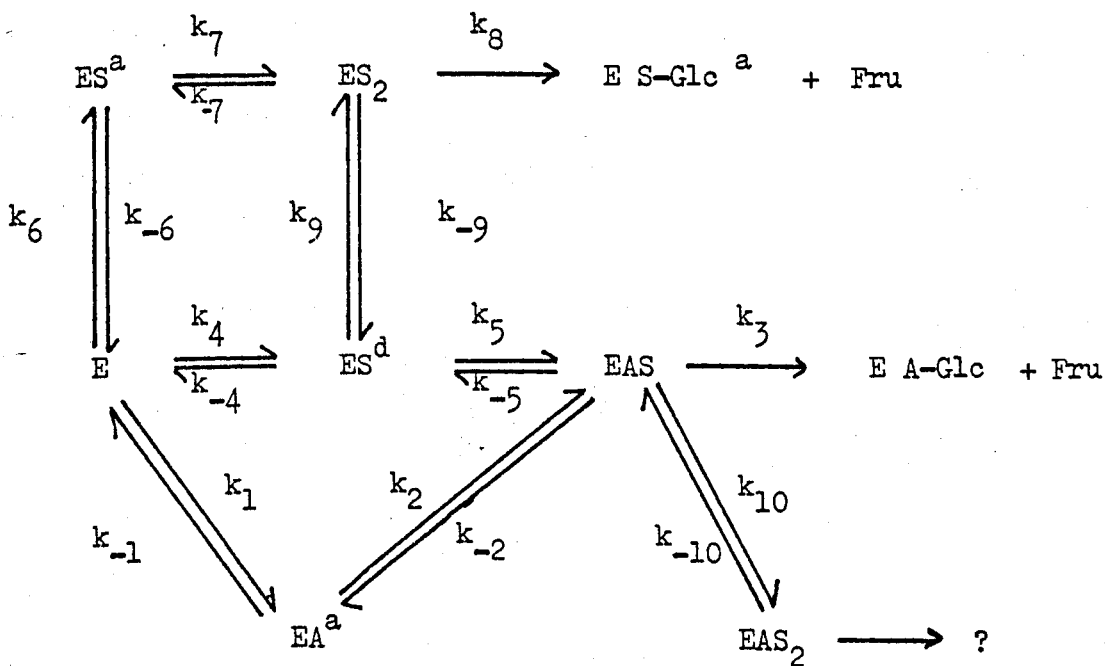
At the maximum velocity obtained (not the V_{max} of the Michaelis-Menten equation; see fig. 4.3), dv/d[S] = 0. Differentiation of both rate equations (Cornish-Bowden, 1976, and Ebert & Schenk, 1968b) at this point gives $[S]^2 = K_m \cdot K_i$. Using the data obtained in this study, values of K_i were calculated. They varied over a wide range, from 160 to 640 mmol. When these values were inserted into either of the two rate equations given above, the calculated curves did not fit the experimental data. The calculated enzyme velocities decreased more slowly with increasing sucrose concentration than the experimental values did. The glucosyltransferases of S. sanguis may need to be described by a more complex reaction scheme than has previously been used. The scheme shown in fig. 4.4 shows the possible pathways of the glucosyltransferase reaction mechanism. The normal reaction pathway of free enzyme molecules in glucan synthesis is probably as follows;



This is because the mechanisms proposed by Robyt et al. (1974) and Ebert and Schenk (1968b) suggest that glucosyl acceptors (such as glucans) are bound to the enzyme molecule before glucosyl donors. It is also likely that the growing glucan chain remains tightly bound to the enzyme molecule (Robyt et al., 1974; Ebert & Schenk, 1968a); in fig. 4.4, k_1 will be very large compared with k_{-1} . Therefore, in the presence of glucan and non-inhibitory concentrations of sucrose, the kinetics of the reaction will be governed by k_2 , k_{-2} and k_3 . The large difference between the behaviour of L. mesenteroides dextran-sucrase and that of S. sanguis glucosyltransferases may be due to the relative magnitudes of the rate constants shown in fig. 4.4. Some of the reactions shown may be insignificant with the L. mesenteroides enzyme, in which case, the rate equation given by Ebert and Schenk (1968b) will be a good approximation. With the S. sanguis enzymes, many of the forward reactions shown may be much faster, relative to the L. mesenteroides enzymes, making the description of the kinetics of this mechanism more complex. The effect of this would not merely be to reduce the value of K_i ; if this were all that happened, the glucosyltransferases would still conform to the rate equation of Ebert and Schenk. In fact, a simple K_i , for binding of sucrose to the acceptor site, may not be sufficient to describe the observed substrate inhibition.

However, although it is useful to consider these theoretical

Fig. 4.4 Postulated reaction mechanism of glucan synthesising glucosyltransferases



Notes

- (i) Superscript 'a' or 'd' indicate that the ligand is bound to the acceptor or donor site respectively.
- (ii) E is Enzyme, S is Substrate (sucrose), A is Glucosyl Acceptor (other than sucrose).
- (iii) A-Glc (or S-Glc) is the reaction product in chain elongation.
- (iv) EAS_2 is the complex formed when a second substrate molecule binds to EAS complex at a third site (as suggested by Ebert & Schenk, 1968a & b).

explanations of the behaviour of the glucosyltransferases of S. sanguis 804, there are other factors which may be important. At high (inhibitory) concentrations of sucrose, any glucan chains synthesised will be shorter and fewer in number. As a result, the efficiency of precipitation by ethanol, in assays for glucan-synthesising activity, may be decreased. These lower concentrations and lower molecular weight of glucans may mean that there are less sites for branching enzyme activity and this too may impair the efficiency of precipitation. If this is the case, the decrease in rate of glucan synthesis, due to substrate inhibition, will appear to be even greater than it really is. This problem might be overcome using carrier glucan (such as dextran) to improve the yield of precipitated glucan in assays for glucosyltransferase activity. If this method was adopted, it might be necessary to use radio-labelled sucrose and to measure the synthesis of radio-labelled glucan in order to avoid the high background which the carrier would give in chemical assays for total precipitated carbohydrate. However, even if the precipitation effect is large enough to affect the mathematical descriptions of the behaviour of the enzymes, the results lose none of their clinical significance. The colonisation of plaque by bacteria is more easily promoted when the glucans present are larger (in mol. wt.), more complex and less soluble (see sections 1.2.3 - 1.2.5). Any decrease in molecular weight or degrees of branching (such as with high sucrose concentrations) can only decrease the adhesiveness of the glucans.

Thus, it has not been possible to explain fully the kinetics of the glucosyltransferases or of the observed substrate inhibition. Moreover, it is not yet possible to say how much this inhibition is lessened by the presence of natural glucosyl acceptors in dental plaque. Nevertheless, it did seem worthwhile to study the oral concentrations of sucrose and to relate the activity of the glucosyltransferases to these. Given that sucrose is common in the diet, it would be useful to know what patterns of sucrose consumption would be least conducive to glucan synthesis in the hope that one could minimise plaque formation.

As shown in section 3.7.3, the salivary concentrations of sucrose produced by various foods are not necessarily a simple function of their sucrose contents. By comparing the oral concentration of sucrose while different beverages are in the mouth (figs. 3.25 and 3.29) with those in the beverages themselves (table 3.8) one can see that the beverages were not greatly diluted by saliva. With solid foods, however, the concentrations of sucrose in the saliva were affected by the physical properties of the foods, especially their rates of dissolution. Lanke (1957) found that the rates of oral clearance of reducing sugar (from foodstuffs themselves or produced by the action of salivary enzymes on them) were similarly dependent upon the physical properties of the foods.

For example, hard boiled sweets dissolved slowly in the mouth (fig. 3.26). This produced moderately high concentrations of sucrose (80-120 mmol/l) and would provide optimal conditions for glucan synthesis over a considerable period of time. Other than removing these sweets from the diet, there seems to be no obvious way of minimising their harmful effects. In contrast, chocolate and toffee

are soft materials and they dissolved in the mouth fairly rapidly (figs. 3.27 and 3.28). As a result, any dental plaque was exposed to sucrose for shorter times than with the hard sweets. Moreover, the sucrose concentrations thus attained were very high, sufficiently so to inhibit glucan synthesis considerably. With these sweets, the periods of maximum glucosyltransferase activity would occur only as sucrose was cleared from the mouth. These periods could be minimised by eating such sweets consecutively. Spasmodic and intermittent consumption of these sweets would expose the teeth to sucrose for longer and acid would be produced over longer periods. The exposure of the plaque to moderate sucrose concentrations for longer, due to the increased number of clearance phases, might also enhance glucan synthesis.

The rates of clearance of oral sucrose following consumption of sweetened drinks such as tea and Coca-Cola (see figs. 3.25 and 3.29) do not seem to be affected much by the concentrations of sucrose in the drinks. The most sensible course would seem to be to avoid beverages which would produce optimal salivary sucrose concentrations for glucan synthesis (i.e. around 70-100 mmol/l). Carbonated and sweetened 'soft' drinks such as Coca-Cola could produce sucrose concentrations sufficient for 50% inhibition of glucosyltransferase activity. Thus, such soft drinks may not be as cariogenic as has previously been supposed. In fact there have been clinical or laboratory trials to suggest that they are significantly more cariogenic than other sucrose-containing beverages. The evidence for such beliefs is largely conjectural.

The preceding paragraphs have attempted to show how the effects of sucrose concentration on glucosyltransferase activity might

be used to help minimise plaque formation and dental caries. In the light of this, it is worth considering other approaches to caries prevention.

There are many methods for prevention of caries which are currently being used or investigated. Fluoridation of public water supplies or of domestic 'table salt' certainly reduces the incidence of dental caries (Backer Dirks et al., 1978; Marthaler et al., 1978) but in many circles this is considered to be unacceptable 'mass medication'. Fluoride may be applied topically as gels, pastes, mouth rinses or tablets; these methods are also successful in inhibiting cariogenesis (various reports, reviewed by Brudevold & Naujoks, 1978) but they are all, to different degrees, 'labour intensive' and give no protection to unerupted teeth unless treatment is maintained until the full permanent dentition is established. The value of fluoride-containing dentifrices is, at present, doubtful (von der Fehr & Møller, 1978). Sealing of tooth fissures with synthetic resins can reduce the incidence by more than 95% (Buonocore, 1971; Horowitz et al., 1971) but, again, this method does not protect unerupted teeth and is labour-intensive. Success with fissure sealants may depend on the skill of the operator in applying and 'curing' the resins.

As well as the approach described in the preceding paragraph, there are many caries-preventive methods specifically designed to inhibit formation or metabolism of dental plaque. Various antiseptics have been investigated and chlorhexidine, the active ingredient of 'Hibitane' and 'Savlon', seems to be particularly effective in preventing plaque formation, dental caries and periodontitis (Parsons, 1974; Loesche, 1975). Unfortunately, chlorhexidine has a particularly

unpleasant, lingering taste. In clinical studies, plaque formation and cariogenesis were inhibited by some antibiotics (Handelman et al., 1962; Loesche et al., 1971; Frostell, 1972). However, the bacteria of dental plaque are widespread and the use of antibiotics could become ineffective, or even dangerous, due to selection of resistant strains of bacteria.

There is an enormous amount of published material (reviewed by Frostell & Ericsson, 1978) on attempts to prevent plaque formation and caries using enzymes or vaccines. The reports are often conflicting or, at best, equivocal. This is because they tend to be based on simplistic premises. For example, dextranase (EC 3.2.1.11, 1,6- α -D-glucanohydrolase) breaks down 1,6- α -D-glucan chains to glucose and isomaltose. This enzyme has been used to inhibit plaque formation but it cannot be expected to degrade plaque glucans completely. These complex glucans also contain 1,3- α -D-glucans which will not be affected by dextranase. Their high degree of branching may produce steric hindrance to attack by dextranase or other glucanases, as suggested by Beeley and Black (1977). Another approach has been to attempt to immunise laboratory animals against caries using vaccines raised against heat-killed S. mutans or S. mutans glucosyltransferases. However, this ignores the fact that S. mutans is not the only species (and not even the only cariogenic species) in dental plaque. It is even doubtful if vaccines against whole plaque would be useful. Bacteria from the mouth are constantly swallowed. It is also likely that the bloodstream is exposed to plaque antigens either through gingivitic bleeding or through traumatic lesions of the oral epithelia. The mouth is capable of resisting infection by oral bacteria. If this

same resistance could be extended to immunity to caries, one would expect this to occur naturally, without artificial intervention; this is not the case.

All the anticaries regimes described above fight a losing battle. In our society, dental caries is primarily due to excessive consumption of refined sucrose (Grenby, 1971). The ideal 'cure' for caries is by prevention, that is, by eliminating sucrose and other refined carbohydrates from the diet; this would also help to reduce the incidence of obesity and lesser degrees of 'overweight'. However, established dietary habits and strong commercial pressures (advertising) make this possibility unlikely, for the near future at least.

Various calorific and non-calorific sweeteners have been suggested as alternatives to sucrose. If actively anticariogenic, these could be incorporated in the diet along with sucrose. If they were merely non-cariogenic, they would have to replace sucrose completely since even small amounts of sucrose can be cariogenic. This would be difficult because many processed foods (especially tinned foods) contain 'hidden' sucrose as a preservative or to 'improve' the taste. There are many artificial, non-calorific sweeteners, such as cyclamate and saccharin. One cannot expect them to help eliminate caries unless they completely replace sucrose in the diet. Even this is not completely satisfactory. None of the artificial sweeteners taste quite the same as sucrose does and, just like alcoholics and drug addicts, the sweet-toothed 'sucrose addict' will probably always tend to revert to eating sucrose.

The most widely investigated calorific sweetener is xylitol. Xylitol is as sweet as sucrose and has a similar, although slightly

drier and cooler taste than sucrose (personal observation). Xylitol is not metabolised by dental plaque in rats (Grunberg et al., 1973) or man (Muhlemann & de Boever, 1973; McFadyen et al., 1976) and, in the absence of sucrose, is not cariogenic (Muhlemann et al., 1970; Scheinin et al., 1975a). Various physiological effects on the 'host' have been attributed to xylitol. Makinen et al. (1976) reported that xylitol stimulated the secretion of salivary lactoperoxidase; this may help prevent dental caries (Koch et al., 1973). Xylitol may stimulate salivary flow; this would increase the electrolyte concentration, pH and buffering power of saliva (Söderling et al., 1970), thus helping to counteract acid production by dental plaque. However, the same effect could apply with many ptyalogogic compounds. Knuuttila and Makinen (1975) reported that the activity of the extracellular glucosyltransferases of S. mutans was slightly inhibited by high concentrations of xylitol. On the basis of this, it has been proposed that xylitol is, in fact, actively anti-cariogenic (Scheinin et al., 1975b).

However, in this study, the glucosyltransferases of S. sanguis 804 were not affected by xylitol over a large concentration range, from 0.01 to 900 mmol/l. Moreover, xylitol did not affect growth, metabolism or glucosyltransferase production of S. sanguis 804 and did not inhibit acid production or carbohydrate metabolism of human dental plaque (Beeley et al., 1978; Hayes & Roberts, 1978). Xylitol did not affect growth or metabolism of S. mutans or A. viscosus (Muhlemann et al., 1977). Xylitol had no effect on plaque formation by S. mutans, in rats, or acid production or formation of 'natural' plaque in man (Muhlemann et al., 1977). All this evidence suggests that xylitol is unlikely to be significantly anti-cariogenic. Thus, partial replacement of dietary sucrose by xylitol would probably not reduce the incidence of caries.

To summarise, the various approaches to prevention of caries are, at worst, unsuccessful or, at best, only a partial solution to the problem. The best methods remain complete avoidance of dietary sucrose (difficult even for those without a sweet tooth) and scrupulous and regular oral hygiene (e.g. by toothbrushing and dental floss) to remove dental plaque.

Sucrose is unlikely to disappear from the diet. However, it has long been known that the incidence of caries in man and laboratory animal is determined by the frequency of sucrose intake not the total amount of sucrose consumed (Gustaffson et al., 1954; Lundqvist, 1957; König et al., 1964; Martinson, 1972; Hartles & Leach, 1975). This was previously explained in terms of the Stephan curve. With the higher frequency of sucrose intake, the dental enamel was exposed to acid production by plaque for longer. There are, however, additional explanations of this. If the frequency of sucrose intake is decreased (e.g. by restricting consumption to set meal-times), then plaque will be exposed to sucrose for shorter periods at much higher concentrations. Acid production by salivary and plaque bacteria is slightly inhibited by high concentrations of sucrose (Sandham & Kleinberg, 1969; Geddes, 1974; Birkhed & Frostell, 1978). As shown in this study, the extracellular glucosyltransferases of S. sanguis 804 are strongly inhibited by high dietary concentrations of sucrose. Thus if the frequency of sucrose consumption is restricted as described above, the resulting high concentrations of sucrose should be sufficient to inhibit acid production and synthesis of extracellular glucans in dental plaque.

Inhibition of acid production and glucan synthesis by high sucrose concentration help explain the clinical findings and show how a sucrose-rich diet can be arranged to minimise the risk of caries.

However, one must be careful not to over-interpret the experimental results. Small amounts of acid and glucan production can still lead to caries and these processes can be extremely active even at low concentrations of sucrose. As shown earlier, the K_m for the glucosyl-transferase system of S. sanguis is about 6-20 mmol/l, corresponding to about 0.2-0.7% (w/v) sucrose. This is about the threshold of taste for sucrose (Moskowitz, 1974) so the rate of glucan synthesis in plaque may be comparatively high even at barely detectable dietary levels of sucrose. In view of this, even the low concentrations of sucrose used to make processed foods or infants' foods appetising seem dangerous.

I have already said that it is unlikely that sucrose will be completely eliminated from the diet. It is however not impossible. The enjoyment of sweet food may be an acquired taste, albeit easily acquired, and infants may be reared with a preference for savoury foods and aversion for sweet ones. Coffee, hopped beer and highly spiced foods are extremely bitter in comparison to many other components of the diet. However, the pressures of advertising and social convention encourage people to acquire the taste for these. Once these tastes have been acquired, they are every bit as compulsive as the taste for sucrose. It should be possible to discourage the taste for sucrose in the same manner. However, at the moment, the public has not been given the incentive to avoid sucrose. A programme of dental health education based on the dangers of dietary sucrose rather than on the dubious advantages of various toothpastes would be a useful start.

I have attempted to use the studies of oral sucrose described in this thesis to predict the patterns of plaque metabolism, in vivo, and to show how the cariogenicity of a sucrose-rich diet might be minimised. It is important to bear in mind, however, that all the experiments on

salivary sucrose concentrations were conducted with one subject. The sucrose concentrations obtained may vary somewhat from subject to subject, especially with solid foods. However, the basic pattern revealed, that rapid dissolution of sweet foodstuffs produces high sucrose concentrations which may inhibit glucosyltransferase activity, probably varies little.

Most of the arguments presented on this subject were based on the salivary concentrations of sucrose. It would, of course, be more meaningful and reliable to base the arguments on the concentrations of sucrose in dental plaque. There are, however, technical difficulties in studying these, as discussed in section 3.7.4. Some of these difficulties have been overcome and preliminary experiments have been carried out (section 3.7.4). The results show that sucrose can penetrate plaque very rapidly (see 3.7.4 and table 3.11). This might be expected from theoretical considerations, since sucrose can diffuse rapidly through plaque (S.G. McNee, personal communication). The sucrose concentration in plaque dropped rapidly after sucrose rinses. This rate of clearance of sucrose from plaque is determined by the rate of metabolism of sucrose by plaque, of diffusion of sucrose from plaque to saliva (and vice versa), and of dilution of plaque sucrose (and oral sucrose, in general) by salivary flow. The conclusions which can be drawn from such preliminary experiments are obviously limited. However, it appears that the rates of clearance of sucrose from saliva and plaque are similar. This may be because, although sucrose can diffuse freely and rapidly from plaque, its rate of clearance will be limited by the concentrations of sucrose remaining in saliva. It is difficult to say how rapidly plaque can metabolise the sucrose and how much this might affect clearance. However,

if the rates of clearance from saliva and plaque are similar, as these experiments suggest, and given the rapid diffusion rates, it is likely that the saliva is a large sucrose reservoir, in excess of the amounts which can be metabolised by plaque.

The discussion above shows that the concentrations of sucrose in dental plaque probably closely follow those in saliva. In order for these arguments to be more conclusive, further studies will be required. In addition to the type of experiments described here (3.7.4), diffusion techniques would be useful. Diffusion studies usually involve measurement of the passage of radioisotope label to or from the sample (e.g. McNee et al., 1979). This technique alone could not measure the rate of penetration of sucrose through plaque; some of the sucrose would be broken down by bacterial metabolism to compounds with different diffusion coefficients from that of sucrose. This problem could be overcome by sampling different depths of a column of plaque (by thin-sectioning) in a diffusion experiment, using radio-labelled sucrose. The amounts of labelled sucrose, and its metabolic products, could be analysed for different depths and diffusion time using thin layer chromatography or gel-filtration. Not only would such experiments show how rapidly sucrose can penetrate plaque, but they would show how rapidly the plaque can metabolise the sucrose. This project is still being investigated.

APPENDIX

To avoid any misunderstandings, I will attempt to clarify the nomenclature of the enzymes which synthesise extracellular glucans in dental plaque.

These highly branched glucans contain both α -1,6 and α -1,3 linkages (see 1.3.3). They comprise water-soluble and insoluble glucans resembling the following;

DEXTRAN Soluble 1,6- α -D-glucan with α -1,3 branches (see fig. A.1)

'MUTAN' Insoluble 1,3- α -D-glucan with α -1,6 branches (see fig. A.2).

There may also be co-polymers intermediate between these two extremes.

Unfortunately, many workers refer to these glucans as 'dextran' or 'dextran-like glucans' and to the enzymes which synthesise them as 'dextransucrase'. However, there is almost certainly more than one enzyme involved in glucan synthesis in plaque.

Dextransucrase is the recommended trivial name for the enzyme which synthesises 1,6- α -D-glucan chains from sucrose, i.e.

EC 2.4.1.5., Sucrose : 1,6- α -D-glucan 6- α -glucosyltransferase.

Dental plaque almost certainly contains not only this enzyme but also the enzyme (or enzymes) which I have called, in this thesis, 'mutan-sucrase', i.e.

EC 2.4.1.x, Sucrose : 1,3- α -D-glucan 3- α -glucosyltransferase.

Robyt and co-workers have suggested that the glucans of L. mesenteroides and S. mutans are branched by alternative reactions of the 'dextransucrases' (discussed in 1.3.4). If distinct branching enzymes exist, they probably act by formation of glycosidic linkages between chains rather than by de novo synthesis of branch chains with

Figure A1 DEXTRAN

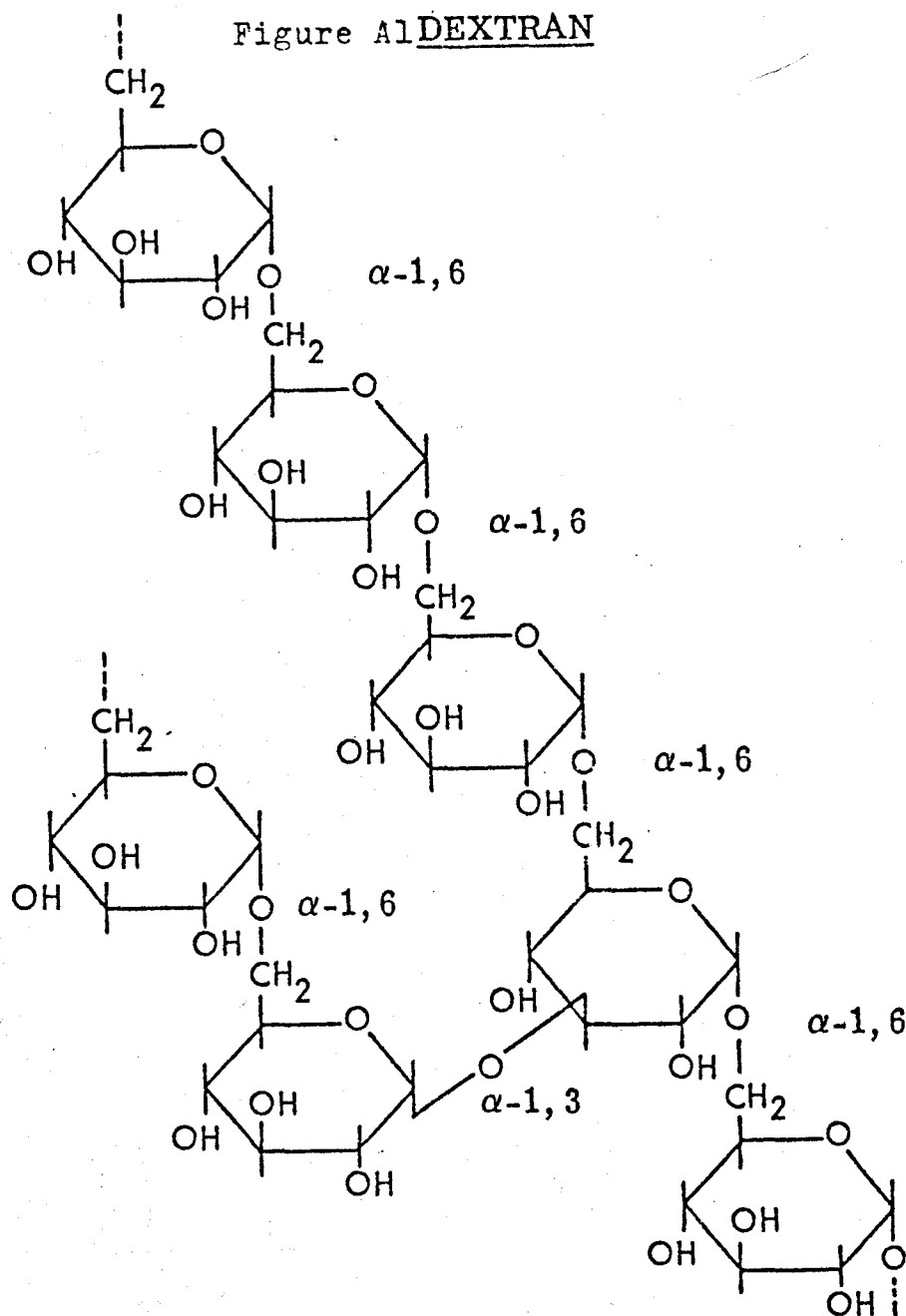
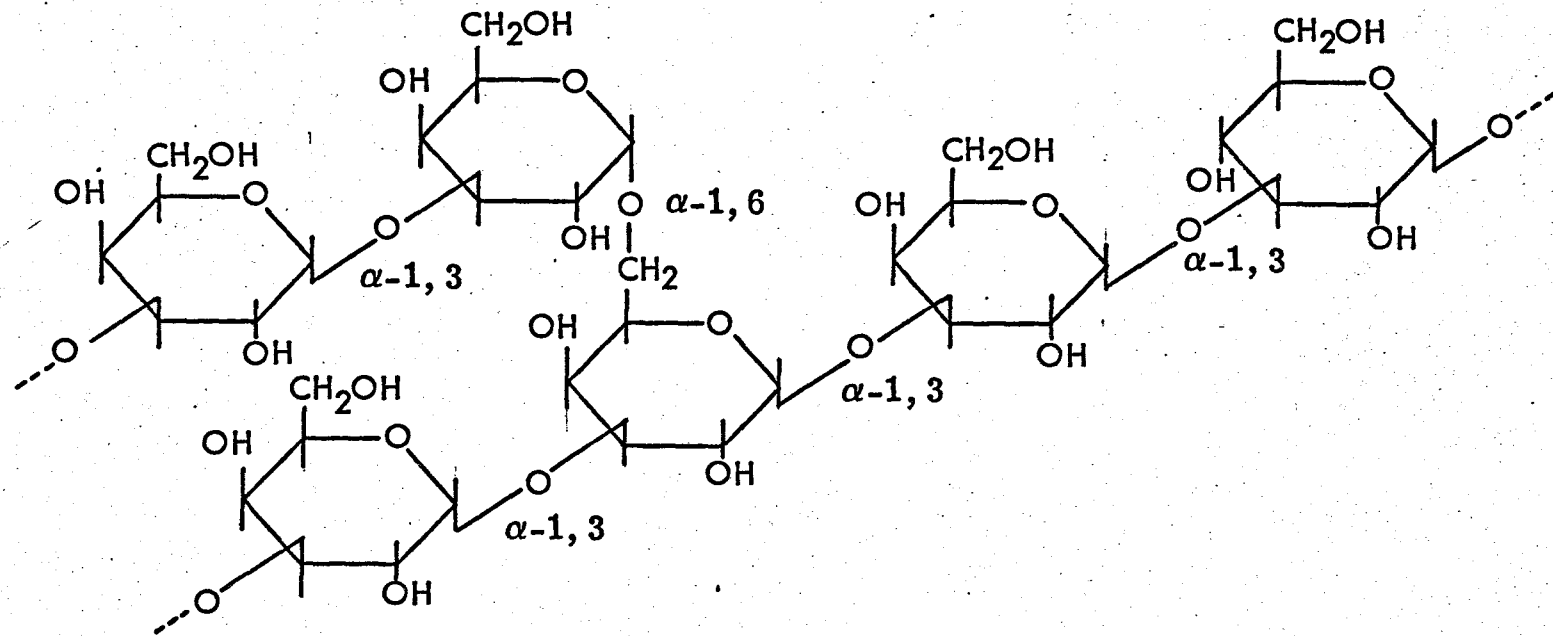


Figure A2 MUTAN



sucrose as glucosyl donor (see 1.3.4). Thus, these enzymes would be glucanotransferases, not glucosyltransferases.

These enzyme systems are still very ill-defined, so I propose to refer to them, in this thesis, merely as 'glucan-synthesising enzymes' or 'glucosyltransferases'.

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